



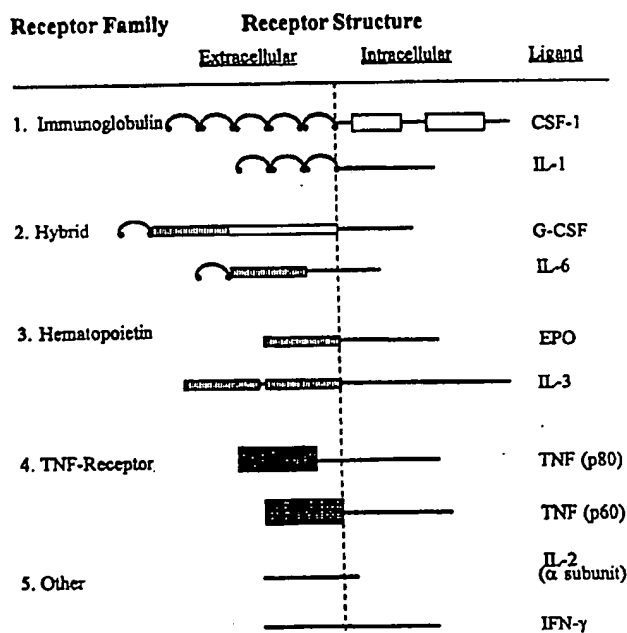
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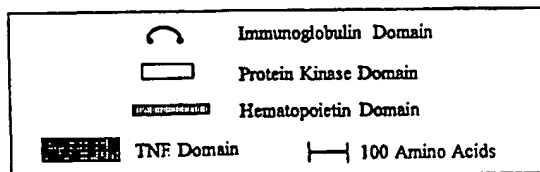
(54) Title: METHOD FOR PREPARING ORPHAN RECEPTOR LIGANDS

## (57) Abstract

Methods for obtaining cells that produce a ligand for an orphan receptor and methods for preparing polynucleotide molecules that encode ligands for orphan receptors are disclosed. The methods utilize growth factor-dependent parent cells that are transfected with a DNA construct encoding an orphan receptor. The transfected cells are exposed to mutagenizing conditions, and the mutagenized cells are cultured under conditions in which cell survival is dependent upon autocrine growth factor production. Progeny cells are recovered and screened to identify those that produce a ligand for the orphan receptor. Polynucleotide molecules encoding the ligand can be prepared from the identified cells.



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Description

## METHOD FOR PREPARING ORPHAN RECEPTOR LIGANDS

Cross-Reference to Related Application

10 The present application is a continuation-in-part of Serial No. 08/196,025, filed February 14, 1994, which application is pending and which is incorporated herein by reference.

Background of the Invention

15 Proliferation and differentiation of cells of multicellular organisms are controlled by hormones and polypeptide growth factors. These diffusable molecules allow cells to communicate with each other and act in concert to form organs and repair damaged tissue.  
20 Examples of hormones and growth factors include the steroid hormones (e.g. estrogen, testosterone), parathyroid hormone, follicle stimulating hormone, the interleukins, platelet derived growth factor (PDGF), epidermal growth factor (EGF), granulocyte-macrophage  
25 colony stimulating factor (GM-CSF), erythropoietin (EPO) and calcitonin.

Hormones and growth factors influence cellular metabolism by binding to receptors. Receptors may be integral membrane proteins that are linked to signalling  
30 pathways within the cell, such as second messenger systems. Other classes of receptors are soluble molecules, such as the transcription factors.

The study of receptor-ligand interactions has revealed a great deal of information about how cells  
35 respond to external stimuli. This knowledge has led to the development of therapeutically important compounds, such as erythropoietin, the colony stimulating factors and

PDGF. Nevertheless, many molecules that control cell growth and development are probably yet to be discovered. In some instances, receptors are known, but their ligands remain to be identified. Discovery of new hormones and growth factors would be aided by the provision of new molecular tools, including receptors, ligands and growth factor-dependent cells. The present invention provides such tools as well as other, related advantages.

10 Summary of the Invention

It is an object of the present invention to provide methods for identifying and isolating ligands for orphan receptors.

15 It is a further object of the present invention to provide methods for cloning polynucleotide molecules encoding ligands for orphan receptors.

It is yet another object of the present invention to provide cell lines that produce ligands for orphan receptors.

20 Within one aspect of the invention, a method for obtaining cells that produce a ligand for an orphan receptor is provided. Broadly stated, the method comprises the steps of (a) providing parent cells, wherein growth of the parent cells is dependent upon an exogenous growth factor; (b) transfecting the parent cells with a DNA construct encoding an orphan receptor to produce transfected cells expressing the orphan receptor encoded by the DNA construct; (c) exposing the transfected cells to mutagenizing conditions to produce mutagenized cells; 25 (d) culturing the mutagenized cells under conditions in which cell survival is dependent upon autocrine growth factor production; (e) recovering progeny cells that survive the culturing step; and (f) screening the progeny cells to identify cells that produce a ligand for the orphan receptor. Within one embodiment, the screening step comprises culturing the progeny cells in the presence 30 35

of an antibody to a known growth factor. Within an alternative embodiment, the screening step comprises culturing the progeny cells in the presence of a soluble form of the orphan receptor. Within another embodiment, the screening step comprises assaying media conditioned by said progeny cells for growth-promoting activity on said parent cells. Within a preferred embodiment, the parent cells do not express detectable levels of the orphan receptor. Within another preferred embodiment, the parent cells are myeloid or lymphoid progenitor cells.

Within another aspect of the invention, a method for preparing a polynucleotide molecule that encodes a ligand for an orphan receptor is provided. The method comprises the steps of (a) providing parent cells, wherein growth of the cells is dependent upon an exogenous growth factor; (b) transfecting the parent cells with a DNA construct encoding an orphan receptor to produce transfected cells expressing the orphan receptor encoded by the DNA construct; (c) exposing the transfected cells to mutagenizing conditions to produce mutagenized cells; (d) culturing the mutagenized cells under conditions in which cell survival is dependent upon autocrine growth factor production; (e) recovering progeny cells that survive the culturing step; (f) screening the progeny cells to identify cells that produce a ligand for the orphan receptor; and (g) preparing polynucleotide molecules encoding the ligand from the identified cells. Polynucleotides that can be prepared according to this method include cDNA, genomic DNA and mRNA.

These and other aspects of the invention will become evident upon reference to the following detailed description and the attached drawing.

#### Brief Description of the Drawings

Figure 1 illustrates the common structural features of five related families of cytokine receptors.

Figure 2 is a partial restriction map of the vector pDX. Symbols used are SV40 ori, the origin of replication from SV40; SV40 E, the SV40 enhancer; MLP, adenovirus major late promoter; L1-3, adenovirus tripartite leader; ss, splicing signals; pA, polyadenylation site.

#### Detailed Description of the Invention

The present invention provides materials and methods that are useful for identifying, isolating and preparing ligands for receptors and polynucleotide molecules encoding those ligands. These materials and methods are particularly useful when the receptor of interest is an "orphan" receptor, that is a receptor for which the identity of the natural ligand is unknown. The present invention thus provides useful tools for identifying, isolating and cloning new growth factors that are useful in, *inter alia*, cell culturing in research and industrial settings, studies of cell physiology and metabolism, and therapeutic intervention in animals including humans.

The term "receptor" is used herein to denote a cell-associated protein that binds to a bioactive molecule (the "ligand") and mediates the effect of the ligand on the cell. Receptors are characterized by a multi-domain structure comprising a ligand-binding domain and an effector domain that is typically involved in signal transduction. Binding of ligand to receptor results in a conformational change in the receptor that causes an interaction between the effector domain and other molecule(s) in the cell. This interaction in turn leads to an alteration in the metabolism of the cell. Metabolic events that are linked to receptor-ligand interactions include gene transcription, phosphorylation, dephosphorylation, increases in cyclic AMP production, mobilization of cellular calcium, mobilization of membrane

lipids, cell adhesion, hydrolysis of inositol lipids and hydrolysis of phospholipids. Receptors can be membrane bound, cytosolic or nuclear; monomeric (e.g. thyroid stimulating hormone receptor, beta-adrenergic receptor) or  
5 multimeric (e.g. PDGF receptor, growth hormone receptor, IL-3 receptor, GM-CSF receptor, G-CSF receptor, erythropoietin receptor and IL-6 receptor).

Receptors are classified into families and superfamilies on the basis of conserved structural  
10 features. It is generally believed that under selective pressure for organisms to acquire new biological functions, new receptor family members arose from duplication of existing receptor genes leading to the existence of multi-gene families. Family members thus  
15 contain vestiges of the ancestral gene, and these characteristic features can be exploited in the isolation and identification of additional family members.

Three of the most well-known receptor superfamilies are the cytokine receptor superfamily, the  
20 seven transmembrane domain (7-TMD) receptor superfamily, and the steroid receptor superfamily. Table 1 provides a partial listing of members of these three receptor superfamilies.

Many cytokine receptors can be placed into one  
25 of five related families on the basis of the structural features shown in Figure 1. All five families are characterized by the presence of an extracellular ligand binding domain and an intracellular domain that are separated by a single transmembrane sequence. Cytokine  
30 receptor structure has been reviewed by Urdal, Ann. Reports Med. Chem. 26:221-228, 1991 and Cosman, Cytokine 5:95-106, 1993.

The 7-TMD receptors are a functionally diverse group encoded by a large gene superfamily. Two  
35 characteristic features of this receptor superfamily are the presence of seven helical transmembrane domains and a

cytoplasmic domain, the latter of which is believed to be responsible for coupling the receptor to G proteins. This superfamily has been reviewed by Lamah et al., Pharm Res. 7:1213-1221, 1990; Hargrave, Curr. Opin. Struct. Biol. 1:575-581, 1991; and Probst et al., DNA and Cell Biol. 11:1-20, 1992.

The steroid receptors may be viewed as ligand-dependent transcription factors. The majority of these receptors appear to contain three domains: a variable, amino-terminal domain; a highly conserved, DNA-binding domain; and a moderately conserved, carboxyl-terminal, ligand-binding domain. The steroid hormone receptor superfamily has been reviewed by Power et al., TIPS 13:318-323, 1992; Parker, Curr. Opin. Cell Biol. 5:499-504, 1993; and McDonnell et al., Bio/Technology 11:1256-1261, 1993. In addition to the known steroid receptors, at least 40 orphan members of this superfamily have been identified (Laudet et al., EMBO J. 11:1003-1013, 1992 and Power et al., *ibid.*).

#### Table 1

##### Cytokine superfamily

###### Immunoglobulin family

CSF-1 receptor

MGF receptor

IL-1 receptor

PDGF receptor

###### Hybrid family

G-CSF receptor

IL-6 receptor

###### Hematopoietin family

Erythropoietin receptor

IL-2 receptor  $\beta$ -subunit

IL-3 receptor

IL-4 receptor

IL-5 receptor



Table 1, continued

IL-7 receptor

IL-9 receptor

GM-CSF receptor  $\alpha$ -subunitGM-CSF receptor  $\beta$ -subunit

IL-6 receptor

growth hormone receptor

TNF receptor

TNF (p80) receptor

TNF (p60) receptor

Other

IL-2 receptor  $\alpha$ -subunitIFN- $\gamma$  receptor7-TMD superfamily

m1 muscarinic acetylcholine receptor

m2 muscarinic acetylcholine receptor

m3 muscarinic acetylcholine receptor

m4 muscarinic acetylcholine receptor

m5 muscarinic acetylcholine receptor

beta 1 adrenergic receptor

beta 2 adrenergic receptor

beta 3 adrenergic receptor

alpha 1 adrenergic receptor

alpha 2A adrenergic receptor

alpha 2B adrenergic receptor

alpha 2-C4 adrenergic receptor

dopamine D1 receptor

dopamine D2 receptor

dopamine D3 receptor

dopamine D4 receptor

dopamine D5 receptor

thrombin receptor

thromboxane receptor

FSH receptor

cannabinoid receptor

gonadotropin receptor

Table 1, continued

thyrotropin receptor  
calcitonin receptor  
parathyroid hormone receptor

5      Steroid superfamily

vitamin D receptor  
glucocorticoid receptor  
mineralocorticoid receptor  
progesterone receptor  
10      androgen receptor  
estrogen receptor  
retinoic acid receptor  
retinoid X receptor

15      Receptors are also classified on the basis of  
common functions. Table 2 presents a listing of receptor  
families grouped according to function. Each tyrosine  
kinase family is represented in Table 2 by a prototypical  
receptor. See Ullrich et al., Nature 308:418-425, 1984;  
20      Ullrich et al., Nature 313:756-761, 1985; Yaden et al.,  
Nature 323:226-232; Hirai et al., Science 238:1717-1720,  
1987; Sanchez-Madrid et al., Proc. Natl. Acad. Sci. USA  
79:7489-7493, 1982; Takeichi, Science 251:1451-1455, 1991;  
Takeichi, Ann. Rev. Biochem. 59:237-252, 1990; and  
25      Cunningham et al., Science 236:799-806, 1987.

Table 2Tyrosine kinase receptors

EGF receptor  
30      insulin receptor  
PDGF receptor  
EPH receptor

Cell adhesion receptors

leukointegrins  
35      cadherin receptors  
immunoglobulin-like receptors

The present invention is based upon a novel process of transfection, mutagenesis and selection that is used to obtain cells, the growth of which is dependent upon an unknown ligand, which produce the ligand. The cells are thus able to grow in the absence of exogenous ligand. Cells obtained in this way can be used, for example, as sources of isolated ligand or nucleic acid molecules encoding the ligand.

The process begins with a cultured parent cell that is dependent on an exogenous growth factor for its proliferation. Suitable cells include animal cells that can be grown in culture. Cultured mammalian cells are preferred. The cells are transfected to produce an orphan receptor. Within a preferred embodiment, the untransfected parent cell does not express detectable levels of the orphan receptor, thereby providing a matched pair of cells with and without the receptor. Many orphan receptors have been identified, and it is anticipated that many more will be found as knowledge of the molecular biology of cells increases. Known orphan receptors include the nuclear receptors COUP-TF1/EAR3, COUP-TF2/ARP-1, EAR-1, EAR-2, TR-2, PPAR1, HNF-4, ERR-1, ERR-2, NGFI-B/Nur77, ELP/SF-1 and, prior to studies disclosed herein, MPL (see reviews by Parker, *ibid.* and Power et al., *ibid.*). A large number of orphan receptors have been identified in the EPH family (Hirai et al., *ibid.*, incorporated herein by reference). HER3 and HER4 (Plowman et al., Proc. Natl. Acad. Sci. USA 90:1746-1750, 1993, incorporated herein by reference) are orphan receptors in the epidermal growth factor receptor family, which may be overexpressed in a number of carcinomas. ILA is a newly identified member of the human nerve growth factor/tumor necrosis factor receptor family (Schwarz et al., Gene 134:295-298, 1993, incorporated herein by reference). An orphan receptor in the insulin receptor family, designated

insulin receptor-related receptor (IRRR) is disclosed by Shier et al. (J. Biol. Chem. 264: 14606-14608, 1989, which is incorporated herein by reference). IRRR is a transmembrane tyrosine kinase. In addition, a number of  
5 orphan tyrosine kinase-type receptors have been found in *Drosophila* (reviewed by Perrimon, Curr. Opin. Cell Biol. 6:260-266, 1994, which is incorporated herein by reference). *Drosophila* orphan receptors are of interest because they present the opportunity for genetic, as well  
10 as biochemical, analysis. Identification of *Drosophila* ligands followed by cloning by homology provides a method for obtaining human or other animal counterparts to the *Drosophila* ligands.

The parent cells are transfected with a DNA  
15 construct encoding the orphan receptor. Such a DNA construct will typically be in the form of a plasmid or virus-derived expression vector. Methods for constructing expression vectors and transfecting cultured cells are known in the art. See, for example, Levinson et al., U.S.  
20 Patent No. 4,713,339; Hagen et al., U.S. Patent No. 4,784,950; Palmiter et al., U.S. Patent No. 4,579,821 and Ringold, U.S. Patent No. 4,656,134, which are incorporated herein by reference in their entirety. In general, a DNA segment encoding the orphan receptor of interest is joined  
25 to expression control sequences in a plasmid or viral vector that may comprise, in addition, one or more origins of replication, one or more selectable markers, enhancers, splice signals or other elements. The DNA construct is inserted into the host cell using conventional methods.  
30 It is preferred to use established cell lines, although primary cultures may also be used.

As noted above, the cell is one in which growth is dependent upon an exogenous growth factor. As used herein, the term "growth factor" denotes a polypeptide  
35 that stimulates proliferation of a cell, the activity of which is mediated by a cell-surface receptor. Examples of

growth factors include the interleukins and colony stimulating factors. Growth factor-dependent myeloid and lymphoid progenitor cells are preferred. These are cells that give rise to differentiated blood cells and that are found in hematopoietic tissue such as bone marrow, spleen and fetal liver. Myeloid and lymphoid precursors are also found in peripheral blood after treatment of an animal with cytokines. Preferred growth factor-dependent cell lines that can be transfected to express orphan receptors include BaF3 (Palacios and Steinmetz, Cell 41: 727-734, 1985; Mathey-Prevot et al., Mol. Cell. Biol. 6: 4133-4135, 1986), FDC-P1 (Hapel et al., Blood 64: 786-790, 1984), and MO7e (Kiss et al., Leukemia 7: 235-240, 1993). Additional growth factor-dependent cell lines are known and available in the art and are disclosed by, for example, Greenberger et al., Proc. Natl. Acad. Sci. USA 80:2931-2935, 1983; Dexter et al., J. Exp. Med. 152:1036-1047, 1980; and Greenberger et al., Virology 105:425-435, 1980. In addition, growth factor-dependent cell lines can be established according to published methods (e.g. Greenberger et al., Leukemia Res. 8: 363-375, 1984; Dexter et al., in Baum et al. Eds., Experimental Hematology Today, 8th Ann. Mtg. Int. Soc. Exp. Hematol. 1979, 145-156, 1980). In a typical procedure, cells are removed from the tissue of interest (e.g. bone marrow, spleen, fetal liver) and cultured in a conventional, serum-supplemented medium, such as RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 15% horse serum and  $10^{-6}$  M hydrocortisone. At one- to two-week intervals non-adherent cells are harvested, and the cultures are fed fresh medium. The harvested, non-adherent cells are washed and cultured in medium with an added source of growth factor (e.g. RPMI 1640 + 10% FBS + 5-20% WEHI-3 conditioned medium as a source of IL-3). These cells are fed fresh medium at one- to two-week intervals and expanded as the culture grows. After several weeks to

several months, individual clones are isolated by plating the cells onto semi-solid medium (e.g. medium containing methylcellulose) or by limiting dilution. Factor dependence of the clones is confirmed by culturing individual clones in the absence of the growth factor. Retroviral infection or chemical mutagenesis can be used to obtain a higher frequency of growth factor-dependent cells.

The orphan receptor-expressing cell is mutagenized to produce a mutant cell. Methods for mutagenizing cells are known in the art and include chemical treatment, exposure to ultraviolet light, exposure to x-rays, and retroviral insertional mutagenesis. Chemical mutagenesis, such as by exposure to ethyl methanesulfonate (EMS), is preferred. Other useful chemical mutagens include nitrosoguanidine, 5-bromouracil, acridine, and aflatoxin. The proportion of mutagenized cells obtained is a function of the strength or amount of mutagenizing agent to which the cells are exposed. A low level of mutagen produces a small proportion of mutant cells. Higher levels of mutagen produce a higher proportion of mutant cells, but also kill more cells. It is therefore necessary to balance mutagenesis with killing so that a reasonable number of mutant cells is obtained. Balancing is generally done empirically by exposing cells to different conditions to establish a killing curve. In general, the cells are exposed to mutagenizing conditions and cultured for one day, after which they are tested for viability according to standard assay methods. Within the present invention, it is preferred to use a level of mutagenesis that results in 20-50% mortality, although one skilled in the art will recognize that this value can be adjusted as necessary, for example if working with a very large number of cells.

The mutagenesis procedure provides a third cell to complement the matched pair of cells with and without

the receptor disclosed above. This set of three cells is a valuable set of tools for use in cloning polynucleotide molecules encoding orphan receptors or their ligands, and for use in related processes.

5           The mutagenized cells are cultured under conditions in which cell survival is dependent upon autocrine growth factor production, that is in the absence of an exogenous growth factor required by the parent cell, to obtain progeny cells. This selection step is very  
10 sensitive because only cells that, through mutagenesis, produce a factor required for their growth or have otherwise become growth factor independent will survive. It is therefore preferred to mutagenize a large number (typically  $1-5 \times 10^7$ ) of cells due to the rarity of such  
15 mutagenic events. Methods for culturing cells are well known in the art. Although the particular conditions will be determined by the specific needs of the particular cell employed, in general the cells will be cultured in a conventional medium containing carbon and nitrogen  
20 sources, minerals, and other nutrients and under suitable conditions of temperature and atmosphere. For example, mammalian cell lines are typically cultured in a buffered cell culture medium containing a carbon source, a nitrogen source, salts, vitamins, antibiotics, amino acids, other  
25 metabolites (e.g. nucleosides, tricarboxylic acid intermediates and lipids) and serum at a temperature of about 37°C in a 5% CO<sub>2</sub> atmosphere. Determination of conditions suitable for the culture of a particular cell is within the level of ordinary skill in the art. See, in  
30 general, Jacoby and Pastan, eds., Meth. Enzymol. vol LVIII, Academic Press, 1979 and Freshney, Culture of Animal Cells: A Manual of Basic Technique, 3rd ed., Wiley-Liss, 1994, which are incorporated herein by reference. Cell culture media are available from commercial suppliers  
35 such as GIBCO BRL (Gaithersburg, MD) and JRH Bioscience (Lexena, KS), or may be prepared according to published

recipes (see, e.g., catalogs of the American Type Culture Collection, Rockville, MD).

Within a preferred embodiment, the cell is cultured in the presence of the exogenous growth factor before it is cultured in the absence of the growth factor. In general, the cells are cultured in the presence of growth factor for about one to seven days prior to being switched to a growth factor-free medium. Within a particularly preferred embodiment, the cells are allowed to recover for about 24 hours in the presence of exogenous growth factor, then plated at 50,000-100,000 cells per well in 24-well culture plates using growth factor-free culture medium. While not wishing to be bound by theory, it is believed that this recovery period allows time for phenotypic expression of the mutagenized gene(s), thereby increasing the likelihood that viable mutagenized cells will be recovered.

The progeny cells are then screened to identify cells that produce a ligand for said cell-surface receptor. This screening step differentiates ligand-producing cells from cells that have undergone other mutagenic events that could be responsible for the apparent autocrine growth stimulation. In general, assays are performed by testing media conditioned by the progeny cells for growth-promoting activity. As used herein, the term "media conditioned by the progeny cells" includes fractions or concentrates of conditioned media. Those skilled in the art will recognize that several types of assays can be used to rule out the presence of unwanted activities and confirm the presence of the activity of interest. Preferred assays for use within the present invention include activity assays using different target cells and neutralization assays. Activity assays are preferred as the primary screen. In a preferred embodiment, progeny cell-conditioned media is assayed for stimulatory activity on transfected and untransfected



parent cells. Stimulatory activity specific for the transfected cells indicates that the ligand for the orphan receptor may be present in the conditioned media. Neutralization assays include assays employing antibodies against potential ligands or soluble forms of the cell-surface receptor of interest. In one embodiment, media conditioned by progeny cells is applied to the transfected parent cells that express the orphan receptor. Antibodies against known growth factors are then added to the media singly and in various combinations. Inhibition of cell growth by one or more of the antibodies indicates that the progeny cells are producing a known ligand. In another embodiment, the transfected parent cells are cultured in progeny cell conditioned media, and a soluble form of the orphan receptor is added to the culture. Inhibition of growth by the soluble receptor indicates that the ligand of interest is being produced by the progeny cells. Those skilled in the art will recognized that variations on these assays can also be employed, such as characterizing the activity by adding antibodies or soluble receptor to the cultured progeny cells.

Within the procedures disclosed above, viable cells are identified by visual inspection of the cultures and/or by viability assay. Many suitable viability assays are known in the art, and include assays for metabolism of a dye such as 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (Mosman, J. Immunol. Meth. 65: 55-63, 1983); 3-(4,5 dimethyl thiazol-2yl)-5-carboxymethoxyphenyl-2H-tetrazolium; 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide; and cyanoditoly-tetrazolium chloride (which are commercially available from Polysciences, Inc., Warrington, PA); mitogenesis assays, such as measurement of incorporation of <sup>3</sup>H-thymidine; dye exclusion assays using, for example, naphthalene black or trypan blue; dye uptake using diacetyl fluorescein; and

chromium release. See, in general, Freshney, Culture of Animal Cells: A Manual of Basic Technique, 3rd ed., Wiley-Liss, 1994, which is incorporated herein by reference.

Cells producing the ligand for the orphan  
5 receptor are a source of polynucleotide molecules that can be used to produce large quantities of the ligand. Useful polynucleotide molecules in this regard include mRNA, cDNA and genomic DNA. For recombinant protein production, cDNA is preferred. Methods for preparing these polynucleotide  
10 molecules are well known in the art. See, for example, Sambrook et al., eds, Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press, 1989; Mullis et al., U.S. Patent No. 4,683,195; and Chirgwin et al., Biochemistry 18: 52-94, 1979. Vectors,  
15 enzymes, and other reagents for use in isolation and cloning of polynucleotide molecules are readily available from commercial suppliers.

Isolated polynucleotide molecules encoding  
ligands for orphan receptors are useful in the production  
20 of polypeptide ligands through the techniques of genetic engineering. In general, the polynucleotide molecule is joined to expression control sequences in a plasmid or viral vector that may comprise, in addition, one or more origins of replication, one or more selectable markers,  
25 enhancers, splice signals or other elements. The vector is inserted into a host cell, which is in turn cultured under suitable conditions so that the ligand is produced. Methods for vector construction, host cell transfection and protein production are well known in the art. See,  
30 for example, Sambrook et al., *ibid*; Welch et al., U.S. Patent No. 5,037,743; Kawasaki, U.S. Patent No. 4,599,311; Kawasaki et al., U.S. Patent No. 4,931,373; McKnight et al., U.S. Patent No. 4,935,349; Levinson et al., U.S. Patent No. 4,713,339; Hagen et al., U.S. Patent No.  
35 4,784,950; Palmiter et al., U.S. Patent No. 4,579,821 and

Ringold, U.S. Patent No. 4,656,134, which are incorporated herein by reference in their entirety.

The invention is further illustrated by the following non-limiting examples.

5

### Examples

#### Example I. Isolation of human MPL receptor cDNAs

Human MPL-P and MPL-K receptor isoform encoding cDNAs were isolated from human erythroid leukemic (HEL) cells (Martin and Papayannopoulou, Science 216: 1233-1235, 1982) by reverse transcriptase polymerase chain reaction (PCR) employing primers made to the published sequence encoding the amino and carboxyl termini of the receptors (Vigon et al., Proc. Natl. Acad. Sci. USA 89: 5640-5644, 1992). Template HEL cell cDNA was synthesized from poly d(T)-selected poly(A)<sup>+</sup> RNA using primer ZC5499 (SEQ ID NO: 3). Thirteen  $\mu$ l of HEL cell poly(A)<sup>+</sup> RNA at a concentration of 1  $\mu$ g/ $\mu$ l was mixed with 3  $\mu$ l of 20 pmole/ $\mu$ l first strand primer ZC5499 (SEQ ID NO: 3). The mixture was heated at 65° C for 4 minutes and cooled by chilling on ice.

First strand cDNA synthesis was initiated by the addition of 8  $\mu$ l of first strand buffer (250 mM Tris-HCl, pH 8.3, 375 mM KCl, 15 mM MgCl<sub>2</sub>) (5x SUPERScript™ buffer; GIBCO BRL, Gaithersburg, MD), 4  $\mu$ l of 100 mM dithiothreitol and 3  $\mu$ l of a deoxynucleotide triphosphate solution containing 10 mM each of dATP, dGTP, dTTP and 5-methyl-dCTP (Pharmacia LKB Biotechnology Inc., Piscataway, NJ). The reaction mixture was incubated at 45°C for 4 minutes followed by the addition of 10  $\mu$ l of 200 U/ $\mu$ l of RNase H<sup>-</sup> reverse transcriptase (SUPERScript™ reverse transcriptase; GIBCO BRL) to the RNA-primer mixture. The reaction was incubated at 45° C for 1 hour followed by an incubation at 50° C for 15 minutes. Sixty  $\mu$ l of TE (10 mM Tris:HCl, pH 8.0, 1 mM EDTA) was added to the reaction followed by chromatography through a 400 pore size gel filtration

column (CHROMA SPIN+TE-400™; Clontech Laboratories Inc., Palo Alto, CA) to remove excess primer.

First strand HEL cell cDNA was used as a template for the amplification of human *MPL-P* receptor cDNA using primers corresponding to the region encoding the amino and carboxyl termini of the receptor protein (Vigon et al., *ibid.*). The primers also each incorporated a different restriction enzyme cleavage site to aid in the directional cloning of the amplified product (ZC5746, SEQ ID NO: 4, containing an *Eco* RI site; ZC5762, SEQ ID NO: 5, containing an *Xho* I site). A 100  $\mu$ l reaction was set up containing 10 ng of template cDNA, 50 pmoles of each primer; 200  $\mu$ M of each deoxynucleotide triphosphate (Pharmacia LKB Biotechnology Inc.); 1  $\mu$ l of 10x PCR buffer (Promega Corp., Madison, WI); and 10 units of Taq polymerase (Roche Molecular Systems, Inc., Branchburg, NJ). The polymerase chain reaction was run for 35 cycles (1 minute at 95° C, 1 minute at 60° C and 2 minutes at 72° C with 1 extra second added to each successive cycle) followed by a 10 minute incubation at 72° C.

Human *MPL-K* receptor cDNA was isolated by polymerase chain reaction amplification from HEL cell cDNA in a manner identical to the *MPL-P* receptor cDNA described above, except primer ZC5762 (SEQ ID NO: 5) was replaced with ZC5742 (SEQ ID NO: 6). PCR primer ZC5742 is specific to the 3' terminus of human *MPL-K* cDNA and incorporated an *Xho* I restriction site to facilitate cloning.

The reaction products were extracted twice with phenol/chloroform (1:1), then once with chloroform and were ethanol precipitated. Following digestion with *Eco* RI and *Xho* I, the products were fractionated on a 0.8% low melt agarose gel (SEA PLAQUE GTG™ low melt agarose; FMC Corp., Rockland, ME). A 1.9 Kb amplified product corresponding to human *MPL-P* receptor cDNA and a 1.7 Kb product corresponding to human *MPL-K* receptor cDNA were

recovered from the excised gel slices by digestion of the gel matrix with  $\beta$ -agarase I (New England Biolabs, Inc., Beverly, MA) followed by ethanol precipitation. The cDNAs were subcloned into the vector pBluescript SK+ (Stratagene Cloning Systems, La Jolla, CA) for validation by sequencing.

Example II. Isolation of Mouse *MPL* Receptor cDNA

Spleens from C57BL/KsJ-db/db mice were removed and immediately placed in liquid nitrogen. Total RNA was prepared from spleen tissue using guanidine isothiocyanate (Chirgwin et al., Biochemistry 18: 52-94, 1979) followed by a CsCl centrifugation step. Spleen poly(A)<sup>+</sup> RNA was isolated using oligo d(T) cellulose chromatography (Aviv and Leder, Proc. Natl. Acad. Sci. U.S.A. 69: 1408-1412, 1972).

Seven and a half  $\mu$ l of poly d(T)-selected poly(A)<sup>+</sup> mouse spleen RNA at a concentration of 1.7  $\mu$ g/ $\mu$ l was mixed with 3  $\mu$ l of 20 pmole/ $\mu$ l first strand primer ZC6091 (SEQ ID NO: 7) containing a Not I restriction site. The mixture was heated at 65° C for 4 minutes and cooled by chilling on ice. First strand cDNA synthesis was initiated by the addition of 8  $\mu$ l of 250 mM Tris-HCl, pH 8.3, 375 mM KCl, 15 mM MgCl<sub>2</sub> (5x SUPERScript™ buffer; GIBCO BRL), 4  $\mu$ l of 100 mM dithiothreitol and 3  $\mu$ l of a deoxynucleotide triphosphate solution containing 10 mM each of dATP, dGTP, dTTP and 5-methyl-dCTP (Pharmacia LKB Biotechnology Inc.) to the RNA-primer mixture. The reaction mixture was incubated at 45° C for 4 minutes followed by the addition of 10  $\mu$ l of 200 U/ $\mu$ l RNase H<sup>-</sup> reverse transcriptase (GIBCO BRL). The efficiency of the first strand synthesis was analyzed in a parallel reaction by the addition of 10  $\mu$ Ci of <sup>32</sup>P- $\alpha$ dCTP to a 10  $\mu$ l aliquot of the reaction mixture to label the reaction for analysis. The reactions were incubated at 45° C for 1 hour followed by an incubation at 50° C for 15 minutes.

Unincorporated  $^{32}\text{P}$ - $\alpha\text{dCTP}$  in the labeled reaction was removed by chromatography on a 400 pore size gel filtration column (CHROMA SPIN + TE-400<sup>TM</sup>; Clontech Laboratories Inc.). Unincorporated nucleotides in the  
5 unlabeled first strand reaction were removed by twice precipitating the cDNA in the presence of 8  $\mu\text{g}$  of glycogen carrier, 2.5 M ammonium acetate and 2.5 volume ethanol. The unlabeled cDNA was resuspended in 50  $\mu\text{l}$  water for use in second strand synthesis. The length of the labeled  
10 first strand cDNA was determined by agarose gel electrophoresis.

Second strand synthesis was performed on first strand cDNA under conditions that promoted first strand priming of second strand synthesis resulting in DNA  
15 hairpin formation. The reaction mixture was assembled at room temperature and consisted of 50  $\mu\text{l}$  of the unlabeled first strand cDNA, 16.5  $\mu\text{l}$  water, 20  $\mu\text{l}$  of 5x polymerase I buffer (100 mM Tris: HCl, pH 7.4, 500 mM KCl, 25 mM  $\text{MgCl}_2$ , 50 mM  $(\text{NH}_4)_2\text{SO}_4$ ), 1  $\mu\text{l}$  of 100 mM dithiothreitol, 2  $\mu\text{l}$  of a  
20 solution containing 10 mM of each deoxynucleotide triphosphate, 3  $\mu\text{l}$  of 5 mM  $\beta\text{-NAD}$ , 15  $\mu\text{l}$  of 3 U/ $\mu\text{l}$  *E. coli* DNA ligase (New England Biolabs Inc., Beverly, MA) and 5  $\mu\text{l}$  of 10 U/ $\mu\text{l}$  *E. coli* DNA polymerase I (Amersham Corp., Arlington Heights, IL). The reaction was incubated at  
25 room temperature for 5 minutes followed by the addition of 1.5  $\mu\text{l}$  of 2 U/ $\mu\text{l}$  RNase H (GIBCO BRL). A parallel reaction in which a 10  $\mu\text{l}$  aliquot of the second strand synthesis mixture was labeled by the addition of 10  $\mu\text{Ci}$   $^{32}\text{P}$ - $\alpha\text{dCTP}$  was used to monitor the efficiency of second strand  
30 synthesis. The reactions were incubated at 15° C for two hours followed by a 15 minute incubation at room temperature. Unincorporated  $^{32}\text{P}$ - $\alpha\text{dCTP}$  in the labeled reaction was removed by chromatography through a 400 pore size gel filtration column (Clontech Laboratories, Inc.)  
35 before analysis by agarose gel electrophoresis. The unlabeled reaction was terminated by two extractions with

phenol/chloroform and a chloroform extraction followed by ethanol precipitation in the presence of 2.5 M ammonium acetate.

5 The single-stranded DNA of the hairpin structure was cleaved using mung bean nuclease. The reaction mixture contained 100  $\mu$ l of second strand cDNA, 20  $\mu$ l of 10x mung bean nuclease buffer (Stratagene Cloning Systems, La Jolla, CA), 16  $\mu$ l of 100 mM dithiothreitol, 51.5  $\mu$ l of water and 12.5  $\mu$ l of a 1:10 dilution of mung bean nuclease  
10 (Promega Corp.; final concentration 10.5 U/ $\mu$ l) in mung bean nuclease dilution buffer. The reaction was incubated at 37° C for 15 minutes. The reaction was terminated by the addition of 20  $\mu$ l of 1 M Tris: HCl, pH 8.0 followed by sequential phenol/chloroform and chloroform extractions as  
15 described above. Following the extractions, the DNA was precipitated in ethanol and resuspended in water.

The resuspended cDNA was blunt-ended with T4 DNA polymerase. The cDNA, which was resuspended in 190  $\mu$ l of water, was mixed with 50  $\mu$ l 5x T4 DNA polymerase buffer  
20 (250 mM Tris:HCl, pH 8.0, 250 mM KCl, 25 mM MgCl<sub>2</sub>), 3  $\mu$ l 0.1 M dithiothreitol, 3  $\mu$ l of a solution containing 10 mM of each deoxynucleotide triphosphate and 4  $\mu$ l of 1 U/ $\mu$ l T4 DNA polymerase (Boehringer Mannheim Corp., Indianapolis, IN). After an incubation of 1 hour at 10° C, the reaction  
25 was terminated by the addition of 10  $\mu$ l of 0.5 M EDTA followed by serial phenol/chloroform and chloroform extractions as described above. The DNA was chromatographed through a 400 pore size gel filtration column (Clontech Laboratories Inc., Palo Alto, CA) to  
30 remove trace levels of protein and to remove short cDNAs less than -400 bp in length. The DNA was ethanol precipitated in the presence of 12  $\mu$ g glycogen carrier and 2.5 M ammonium acetate and was resuspended in 10  $\mu$ l of water. Based on the incorporation of <sup>32</sup>P- $\alpha$ dCTP, the yield  
35 of cDNA was estimated to be -2  $\mu$ g from a starting mRNA template of 12.5  $\mu$ g.

*Eco* RI adapters were ligated onto the 5' ends of the cDNA to enable cloning into a lambda phage vector. A 10  $\mu$ l aliquot of cDNA ( $\sim 2\mu$ g) and 10  $\mu$ l of 65 pmole/ $\mu$ l of *Eco* RI adapter (Pharmacia LKB Biotechnology Inc.) were mixed with 2.5  $\mu$ l 10x ligase buffer (Promega Corp.), 1  $\mu$ l of 10 mM ATP and 2  $\mu$ l of 15 U/ $\mu$ l T4 DNA ligase (Promega Corp.). The reaction was incubated overnight ( $\sim 18$  hours) at a temperature gradient of 0° C to 18° C. The reaction was further incubated overnight at 12° C. The reaction was terminated by the addition of 75  $\mu$ l of water and 10  $\mu$ l of 3 M Na acetate, followed by incubation at 65° C for 30 minutes. After incubation, the cDNA was extracted with phenol/chloroform and chloroform as described above and precipitated in the presence of 2.5 M ammonium acetate and 1.2 volume of isopropanol. Following centrifugation, the cDNA pellet was washed with 70% ethanol, air dried and resuspended in 89  $\mu$ l water.

To facilitate the directional cloning of the cDNA into a lambda phage vector, the cDNA was digested with *Not* I, resulting in a cDNA having 5' *Eco* RI and 3' *Not* I cohesive ends. The *Not* I restriction site at the 3' end of the cDNA had been previously introduced through primer ZG6091 (SEQ ID NO: 7). Restriction enzyme digestion was carried out in a reaction containing 89  $\mu$ l of cDNA described above, 10  $\mu$ l of 6 mM Tris:HCl, 6 mM MgCl<sub>2</sub>, 150 mM NaCl, 1 mM DTT (10x D buffer; Promega Corp., Madison, WI) and 1  $\mu$ l of 12 U/ $\mu$ l *Not* I (Promega Corp.). Digestion was carried out at 37° C for 1 hour. The reaction was terminated by serial phenol/chloroform and chloroform extractions. The cDNA was ethanol precipitated, washed with 70% ethanol, air dried and resuspended in 20  $\mu$ l of 1x gel loading buffer (10 mM Tris:HCl, pH 8.0, 1 mM EDTA, 5% glycerol and 0.125% bromophenol blue).

The resuspended cDNA was heated to 65°C for 5 minutes, cooled on ice and electrophoresed on a 0.8% low



melt agarose gel (SEA PLAQUE GTG™ low melt agarose; FMC Corp.). Unincorporated adapters and cDNA below 1.6 Kb in length were excised from the gel. The electrodes were reversed, and the cDNA was electrophoresed until concentrated near the lane origin. The area of the gel containing the concentrated cDNA was excised and placed in a microfuge tube, and the approximate volume of the gel slice was determined. An aliquot of water (300  $\mu$ l) approximately three times the volume of the gel slice was added to the tube, and the agarose was melted by heating to 65° C for 15 minutes. Following equilibration of the sample to 42° C, 10  $\mu$ l of 1 U/ $\mu$ l  $\beta$ -agarase I (New England Biolabs, Inc.) was added, and the mixture was incubated for 90 minutes to digest the agarose. After incubation, 40  $\mu$ l of 3 M Na acetate was added to the sample, and the mixture was incubated on ice for 15 minutes. The sample was centrifuged at 14,000 x g for 15 minutes at room temperature to remove undigested agarose. The cDNA in the supernatant was ethanol precipitated, washed in 70% ethanol, air-dried and resuspended in 37  $\mu$ l of water for the kinase reaction to phosphorylate the ligated *Eco* RI adapters.

To the 37  $\mu$ l cDNA solution described above was added 10  $\mu$ l 10x ligase buffer (Stratagene Cloning Systems), and the mixture was heated to 65° C for 5 minutes. The mixture was cooled on ice, and 5  $\mu$ l 10 mM ATP and 3  $\mu$ l of 10 U/ $\mu$ l T4 polynucleotide kinase (Stratagene Cloning Systems) were added. The reaction was incubated at 37°C for 45 minutes and was terminated by heating to 65° C for 10 minutes followed by serial extractions with phenol/chloroform and chloroform. The phosphorylated cDNA was ethanol precipitated in the presence of 2.5 M ammonium acetate, washed with 70% ethanol, air dried and resuspended in 12.5  $\mu$ l water. The concentration of the phosphorylated cDNA was estimated to be ~40 fmole/ $\mu$ l.

The resulting cDNA was cloned into the lambda phage vector  $\lambda$ Excell (Pharmacia LKB Biotechnology Inc.), purchased predigested with Eco RI and Not I and dephosphorylated. Ligation of cDNA to vector was carried out in a reaction containing 2  $\mu$ l of 20 fmole/ $\mu$ l prepared  $\lambda$ Excell phage arms, 4  $\mu$ l of water, 1  $\mu$ l 10x ligase buffer (Promega Corp.), 2  $\mu$ l of 40 fmole/ $\mu$ l cDNA and 1  $\mu$ l of 15 U/ $\mu$ l T4 DNA ligase (Promega Corp.). Ligation was carried out at 4° C for 48 hours. Approximately 50% of the ligation mixture was packaged into phage using Gigapack II Gold packaging extract (Stratagene Cloning Systems) according to the directions of the vendor. The resulting cDNA library contained over  $1.5 \times 10^7$  independent recombinants with background levels of insertless phage of less than 1.5%.

A  $^{32}$ P-labeled human MPL-K receptor cDNA probe was used to isolate mouse MPL receptor cDNA from the mouse spleen cDNA phage library. The cDNA library was plated on Sure strain of *E. coli* cells (Stratagene Cloning Systems) at a density of 40,000 to 50,000 PFU/150 mm diameter plate. Phage plaques from thirty-three plates were transferred onto Hybond N<sup>TM</sup> filters (Amersham Corp., Arlington Heights, IL) and processed according to the directions of the manufacturer. The processed filters were baked for 2 hours at 80° C in a vacuum oven followed by several washes at 70° C in wash buffer (0.25 x SSC, 0.25% SDS, 1 mM EDTA) and prehybridized overnight at 65° C in hybridization solution (5x SSC, 5x Denhardt's solution, 0.1% SDS, 1 mM EDTA and 100  $\mu$ g/ml heat denatured salmon sperm DNA) in a hybridization oven (model HB-2; Techne Inc., Princeton, NJ). Following prehybridization, the hybridization solution was discarded and replaced with fresh hybridization solution containing approximately  $2 \times 10^6$  cpm/ml of  $^{32}$ P-labeled human MPL-K cDNA prepared by the use of a commercially available labeling kit (MEGAPRIME<sup>TM</sup> kit; Amersham Corp., Arlington Heights, IL). The probe

was denatured at 98° C for 5 minutes before being added to the hybridization solution. Hybridization was at 65° C overnight. The filters were washed at 55° C in wash buffer (0.25 x SSC, 0.25% SDS, 1 mM EDTA) and were  
5 autoradiographed with intensifying screens for 4 days at -70° C on XAR-5 film (Kodak Inc., Rockchester, NY). Employing the autoradiograph as template, agar plugs were recovered from regions of the plates corresponding to primary signals and were soaked in SM (0.1 M NaCl; 50 mM  
10 Tris:HCl, pH 7.5, 0.02% gelatin) to elute phage for plaque purification. Seven plaque-purified phages were isolated that carried inserts hybridizing to the human *MPL-K* receptor probe. The phagemids contained within the  $\lambda$  Excell phage were recovered using the *in vivo*  
15 recombination system in accordance with the directions of the vendor. The identity of the cDNA inserts was confirmed by DNA sequencing.

The isolated clones encoded a protein exhibiting a high degree of sequence identity to human *MPL-P*  
20 receptor and to a recently reported mouse *MPL* receptor (Skoda et al., EMBO J. 12: 2645-2653, 1993). The seven clones fell into two classes differing from each other by three clones having a deletion of sequences encoding a stretch of 60 amino acid residues near the N-terminus.  
25 The cDNA encoding the protein without the deletion was referred to as mouse Type I *MPL* receptor cDNA. Type II receptor cDNA lacked sequences encoding Type I receptor residues 131 to 190 of SEQ ID NO: 17. In addition, Type I and II receptors differed from the reported mouse *MPL*  
30 receptor sequence (Skoda et al., *ibid.*) by the presence of a sequence encoding the amino acid residues Val-Arg-Thr-Ser-Pro-Ala-Gly-Glu (SEQ ID NO: 9) inserted after amino acid residue 222 and by a substitution of a glycine residue for serine at position 241 (positions refer to the  
35 Type I mouse receptor).

Type I and II mouse *MPL* receptor cDNAs were subcloned into vector pHZ-1 for expression in mammalian cells. Plasmid pHZ-1 is an expression vector that may be used to express protein in mammalian cells or in a frog oocyte translation system from mRNAs that have been transcribed *in vitro*. The pHZ-1 expression unit comprises the mouse metallothionein-1 promoter, the bacteriophage T7 promoter flanked by multiple cloning banks containing unique restriction sites for insertion of coding sequences, the human growth hormone terminator and the bacteriophage T7 terminator. In addition, pHZ-1 contains an *E. coli* origin of replication; a bacterial beta lactamase gene; a mammalian selectable marker expression unit comprising the SV40 promoter and origin, a neomycin resistance gene and the SV40 transcription terminator. To facilitate directional cloning into pHZ-1, a polymerase chain reaction employing appropriate primers was used to create an *Eco* RI site and a *Xho* I site upstream from the translation initiation codon and downstream from the translation termination codon, respectively. The polymerase chain reaction was carried out in a mixture containing 10  $\mu$ l 10x ULTMA™ DNA polymerase buffer (Roche Molecular Systems, Inc., Branchburg, NJ), 6  $\mu$ l of 25 mM  $MgCl_2$ , 0.2  $\mu$ l of a deoxynucleotide triphosphate solution containing 10 mM each of dATP, dGTP, dTTP and dCTP (Pharmacia LKB Biotechnology Inc.), 2.5  $\mu$ l of 20 pmole/ $\mu$ l primer ZC6603 (SEQ ID NO: 8), 2.5  $\mu$ l of 20 pmole/ $\mu$ l primer ZC5762 (SEQ ID NO: 5), 32.8  $\mu$ l of water, 1  $\mu$ l of an early log phase bacterial culture harboring either a Type I or a Type II mouse *MPL* receptor plasmid and 1  $\mu$ l of 6 U/ $\mu$ l DNA polymerase (ULTMA™ polymerase; Roche Molecular Systems, Inc., Branchburg, NJ). AmpliWax™ (Roche Molecular Systems, Inc.) was employed in the reaction according to the directions of the vendor. The polymerase chain reaction was run for 25 cycles (1 minute at 95° C, 1 minute at 55° C and 3 minutes at 72° C) followed by a 10

minute incubation at 72° C. The amplified products were serially extracted with phenol/chloroform and chloroform; then ethanol precipitated in the presence of 6 µg glycogen carrier and 2.5 M ammonium acetate. The pellets were  
5 resuspended in 87 µl of water to which was added 10 µl of 10 x H buffer (Boehringer Mannheim Corp.), 2 µl of 10 U/µl *Eco* RI (Boehringer Mannheim) and 1 µl of 40 U/µl *Xho* I (Boehringer Mannheim Corp.). Digestion was carried out at 37° C for 1 hour. The reaction was terminated by heating  
10 to 65° C for 15 minutes and chromatographed through a 400 pore size gel filtration column (CHROMA SPIN + TE-400™; Clontech Laboratories Inc.).

The isolated receptor inserts described above were ligated into *Eco* RI and *Xho* I digested and  
15 dephosphorylated pHZ-1 vector. The ligation reaction contained 1 µl of 50 ng/µl prepared pHZ-1 vector, 5 µl of 5 ng/µl cDNA insert, 2 µl of 10x ligase buffer (Promega Corp.), 11.75 µl water and 0.25 µl of 4 U/µl T4 DNA ligase (Stratagene Cloning Systems). Ligation was carried out at  
20 10° C overnight. The ligated DNAs were transfected into *E. coli* (MAX EFFICIENCY DH10B™ competent cells; GIBCO BRL) in accordance with the vendor's directions. The validity of Type I and Type II mouse *MPL* and human *MPL-P* receptor inserts in pHZ-1 was confirmed by DNA sequencing. The  
25 resulting plasmids pSLmpl-8 and pSLmpl-9 carried the mouse Type II and Type I *MPL* receptor cDNAs, respectively. Plasmid pSLmpl-44 carried the human *MPL-P* cDNA insert.

### Example III. Construction of BaF3 Cell Lines Expressing 30 MPL Receptors

BaF3, an interleukin-3 dependent pre-lymphoid cell line derived from murine bone marrow (Palacios and Steinmetz, Cell 41: 727-734, 1985; Mathey-Prevot et al., Mol. Cell. Biol. 6: 4133-4135, 1986), was maintained in  
35 complete media (RPMI 1640 medium (JRH Bioscience Inc., Lenexa, KS) supplemented with 10% heat-inactivated fetal

calf serum, 4% conditioned media from cultured WEHI-3 cells (Becton Dickinson Labware, Bedford, MA), 2mM L-glutamine, 2-mercaptoethanol (1:280,000 final conc.) and PSN antibiotics (GIBCO BRL)). Cesium chloride purified plasmids pSLmpl-8, pSLmpl-9 and pSLmpl-44 were linearized at the *Nde* I site prior to electroporation into BaF3 cells. BaF3 cells for electroporation were washed once in RPMI 1640 media and resuspended in RPMI 1640 media at a cell density of  $10^7$  cells/ml. One ml of resuspended BaF3 cells was mixed with 30  $\mu$ g of each of the linearized plasmid DNAs and transferred to separate disposable electroporation chambers (GIBCO BRL). Following a 15 minute incubation at room temperature the cells were given two serial shocks (800  $\mu$ Fad/300 V.; 1180  $\mu$ Fad/300 V.) delivered by an electroporation apparatus (CELL-PORATOR™; GIBCO BRL). After a 5 minute recovery time, the electroporated cells were transferred to 10 ml of complete media and placed in an incubator for 15-24 hours (37° C, 5% CO<sub>2</sub>). The cells were then spun down and resuspended in 10 ml of complete media containing 1600  $\mu$ g/ml G418 and plated at limiting dilutions in 96-well tissue culture plates to isolate G418-resistant clones. Expression of MPL receptors in G418-resistant BaF3 clones was inferred by Northern blot analysis of BaF3 mRNA for the presence of *MPL* receptor transcript. A cell line designated BaF3/MPLR1.1 was found to express high levels of Type I mouse *MPL* receptor mRNA and was used for subsequent assay for *MPL* ligand activity in conditioned media of transfected BHK 570 cells. A BaF3 cell line expressing Type II receptor mRNA was designated as BaF3/MPLR2.

#### Example IV. Production of Soluble Mouse MPL Receptor

A mammalian expression plasmid encoding soluble mouse Type I *MPL* receptor (pLDmpl-53) was produced by combining DNA segments from pSLmpl-9, a mammalian expression plasmid containing the cDNA encoding full-

length mouse Type I *MPL* receptor described above, with a DNA segment from pSLmpl-26, an expression plasmid constructed to produce the soluble mouse Type I *MPL* receptor in bacteria.

5 A cDNA segment encoding mouse Type I *MPL* soluble receptor was isolated by PCR employing primers ZC6704 (SEQ ID NO: 10) and ZC6703 (SEQ ID NO: 11) using full-length receptor plasmid pSLmpl-9 as template. To facilitate directional cloning, primers ZC6704 and ZC6703  
10 incorporated *Eco* RI and *Xho* I restriction sites at their respective 5' ends. Primer ZC6703 also encoded an inframe consensus target sequence for protein kinase to enable *in vitro* labeling of the purified soluble receptor with  $^{32}\text{P}$   $\gamma$ -ATP (Li et al., Proc. Natl. Acad. Sci. U.S.A. 86: 558-562, 1989). The PCR was carried out in a mixture containing 10  
15  $\mu\text{l}$  10x ULTMA<sup>TM</sup> DNA polymerase buffer (Roche Molecular Systems, Inc.), 6  $\mu\text{l}$  of 25 mM  $\text{MgCl}_2$ , 0.2  $\mu\text{l}$  of a deoxynucleotide triphosphate solution containing 10 mM each of dATP, dGTP, dTTP and dCTP (Pharmacia LKB  
20 Biotechnology Inc.), 11  $\mu\text{l}$  of 4.55 pmole/ $\mu\text{l}$  primer ZC6704 (SEQ ID NO: 10), 21  $\mu\text{l}$  of 2.43 pmole/ $\mu\text{l}$  primer ZC6703 (SEQ ID NO: 11), 50.3  $\mu\text{l}$  of water, 1  $\mu\text{l}$  50 ng/ $\mu\text{l}$  *Hind* III and *Xba* I digested pSLmpl-9 and 1  $\mu\text{l}$  of 6 U/ $\mu\text{l}$  ULTMA<sup>TM</sup> DNA polymerase (Roche Molecular Systems, Inc.). AmpliWax<sup>TM</sup>  
25 (Roche Molecular Systems, Inc.) was employed in the reaction according to the directions of the vendor. The polymerase chain reaction was run for 3 cycles (1 minute at 95° C, 1 minute at 50° C and 2 minutes at 72° C) followed by 11 cycles at increased hybridization  
30 stringency (1 minute at 95° C, 30 seconds at 55° C and 2 minutes at 72° C) followed by a 10 minute incubation at 72° C. The amplified product was serially extracted with phenol/chloroform and chloroform followed by chromatography through a 400 pore size gel filtration  
35 column (Clontech Laboratories, Inc.). The PCR product was ethanol precipitated in the presence of 20  $\mu\text{g}$  glycogen

carrier and 2.5 M ammonium acetate. The pellet was resuspended in 32  $\mu$ l of water. To 16  $\mu$ l of the resuspended PCR product was added 2  $\mu$ l 10x H buffer (Boehringer Mannheim Corp.), 1  $\mu$ l of 10 U/ $\mu$ l Eco RI (Boehringer Mannheim Corp.) and 1  $\mu$ l of 40 U/ $\mu$ l Xho I (Boehringer Mannheim Corp.). Digestion was carried out at 37° C for 1 hour. Digestion was terminated by heating to 65° C for 15 minutes and was purified on a 0.7% low-melt agarose gel. Fragment recovery from low-melt agarose was done by digestion of the gel matrix with  $\beta$ -agarase I (New England Biolabs).

The resulting PCR product encoded the N-terminal extracellular domain of mouse Type I MPL receptor (residues 27 to 480 of SEQ ID NO: 17). In the absence of the putative receptor trans-membrane domain (residues 483 to 504 of SEQ ID NO: 17) the expressed protein is expected to be secreted in the presence of a suitable signal peptide. A mouse Type II soluble MPL receptor encoding cDNA was obtained using the PCR conditions described above except that pSLmpl-8 was used as template. The validity of both receptor fragments was confirmed by DNA sequencing.

The soluble mouse Type I and Type II MPL receptor encoding DNA fragments were cloned into Eco RI and Xho I digested vector pOmpA2-5 to yield pSLmpl-26 and pSLmpl-27, respectively. Plasmid pOmpA2-5 is a modification of pOmpA2 (Ghrayab et al., EMBO J. 3: 2437-2442, 1984), a bacterial expression vector designed to target the recombinant protein to the periplasmic space. pOmpA2-5 was constructed by replacement of a 13 bp sequence between the Eco RI and Bam HI sites of pOmpA2 with a synthetic 42 bp sequence. The sequence was created by annealing of two 42 nt complementary oligonucleotides (ZC6707, SEQ ID NO: 12; ZC 6706, SEQ ID NO: 13), which when base paired formed Eco RI and Bam HI cohesive ends, facilitating directional cloning into Eco RI and Bam HI



digested pOmpA2. Within the inserted sequence is an *Xho* I site inframe with respect to a bacterial leader sequence and to the mouse MPL soluble receptor encoding cDNAs described above, as well as an inframe tract of 6 histidine codons located 3' of the *Xho* I site to enable the recombinant protein to be purified by metal chelation affinity chromatography (Houchuli et al., Bio/Technol. 6: 1321-1325, 1988). Following the sequence encoding the histidine tract was an inframe termination codon. The validity of the pOmpA2-5, pSLmpl-26 and pSLmpl-27 was confirmed by DNA sequencing.

pLDmpl-53, a mammalian expression plasmid producing soluble mouse Type I MPL receptor, was constructed by combining DNA segments from pSLmpl-9 and pSLmpl-26 into expression vector pHZ-200 (pHZ-1 in which a dihydrofolate reductase sequence was substituted for the neomycin resistance gene). The 1164 bp *Eco* RI/*Bam* HI cDNA fragment from pSLmpl-9 replaced the mammalian signal sequence deleted during the construction of bacterial expression plasmid pSLmpl-26. The 416 bp *Bam* HI fragment from pSLmpl-26 supplied the coding sequence for the carboxy-terminal portion of the soluble MPL receptor, the kinase labeling domain, the poly-histidine tract and the translation terminator. The two fragments were gel purified and cloned into the *Eco* RI/*Bam* HI sites of pBluescript KS+ (Stratagene Cloning Systems) to yield plasmid pBS8.76LD-5. Correct orientation of the the 416 bp pSLmpl-26 derived *Bam* HI fragment with respect to the 1164 bp pSLmpl-9 derived *Eco* RI/*Bam* HI fragment in pBS8.76LD-5 was determined by PCR using primers ZC 6603 (SEQ ID NO: 8) and ZC 6703 (SEQ ID NO: 11). The *Xba* I site within the poly-linker sequence of pBS8.76LD-5 enabled the reconstituted receptor cDNA to be excised as an 1.5 kb *Eco* RI/*Xba* I fragment for cloning into pHZ-200 following digestion of the vector with *Eco* RI and *Xba* I.

The resulting mammalian expression plasmid, pLDmpl-53, was prepared in large scale for transfection into BHK cells.

Twenty micrograms of purified pLDmpl-53 plasmid was transfected into BHK 570 cells using the calcium phosphate precipitation method. After 5 hours, the cells were shocked with 15% glycerol for 3 minutes to facilitate uptake of DNA. Fresh growth media was added overnight. The following day the cells were split at various dilutions, and selection media containing 1  $\mu$ M methotrexate was added. After approximately two weeks, discrete, methotrexate-resistant colonies were visible. Resistant colonies were either pooled or maintained as distinct clones. Spent media from the pooled colonies was immediately tested for presence of soluble MPL receptor protein.

Soluble MPL receptor protein was isolated through the interaction of the poly-histidine tract present on the carboxy-terminal of the protein with a metal chelation resin containing immobilized  $\text{Ni}^{2+}$  (HIS-BIND<sup>TM</sup>; Novagen, Madison, WI). Serum-free spent culture media from the pLDmpl-53 pool was passed over the resin, and bound protein was eluted with imidazole. SDS-PAGE analysis revealed a single band at ~67 kDa. This protein was subjected to N-terminal amino acid analysis and confirmed to be mouse MPL receptor.

Soluble mouse MPL receptor was purified from a pool of BHK transfectants, which had been transfected with soluble mouse Type I MPL receptor expressing plasmid, pLDmpl-53. The purified soluble receptor was immobilized on CNBr-activated SEPHAROSE<sup>TM</sup> 4B (Pharmacia LKB Biotechnology, Inc.) matrix essentially as directed by the manufacturer and used for affinity purification of the MPL activity in conditioned media of 24-11-5 cells. The affinity matrix was packed in a XK16 column (Pharmacia LKB Biotechnology Inc.). Conditioned media from 24-11-5 cells were concentrated on a 10 Kd cut off Hollow Fiber Membrane

(A/G Technology Corp., Needham, MA) and loaded onto the bottom of the MPL receptor affinity column at a flow rate of 1 ml/minute. The column was washed with phosphate buffered saline (PBS) containing 0.5 M NaCl and 0.01% sodium azide. MPL activity was eluted from the column with 3M potassium thiocyanate (Sigma Chemical Company, St. Louis, MO) at a flow rate of 0.5 ml/minute. Potassium thiocyanate was removed by dialysis against PBS. Active fractions were identified by MTT proliferation assay.

Example V. Isolation and Characterization of a MPL Receptor Ligand Expressing Cell Line

BaF3/MPLR1.1 cells are IL-3 dependent cells expressing a stably transfected Type I mouse MPL receptor. A mutagenesis and selection scheme was devised to isolate cell lines expressing the MPL receptor ligand by mutagenizing BaF3/MPLR1.1 cells, and selecting for autocrine growth in the absence of exogenous IL-3.

Approximately  $1.2 \times 10^6$  BaF3/MPLR1.1 cells were pelleted and washed with GM (RPMI 1640 media supplemented with 2-mercaptoethanol (1:240,000 final concentration), 2 mM L-glutamine, 110  $\mu$ g/ml sodium pyruvate, 50  $\mu$ g/ml G418 and 10% heat inactivated fetal bovine serum). The cells were resuspended in 2 ml of GM containing 0.15% (v/v) of the mutagen 2-ethylmethanesulfonate (EMS) and incubated for 2 hours at 37°C. After incubation, the cells were washed once in PBS and once in GM and plated onto 10 cm plates at density of approximately 40,000 cells/ml in GM supplemented with 5% WEHI-3 conditioned media (Becton Dickinson Labware, Bedford, MA) as a source of IL-3. The cells were allowed a recovery period of seven days incubated at 37°C under 5% CO<sub>2</sub> before selection for IL-3 independent growth. Following the recovery period, the culture was dense with viable cells. The cells were washed with GM and were cultured in GM in the absence of WEHI-3 conditioned media. After eleven days of

selection, small numbers of viable cells were observed. The viable cell density of the IL-3 independent culture was estimated to be 250 cells/ml. One ml of the IL-3 independent culture was plated onto each of 19 wells of a 24-well culture plate for further characterization.

Conditioned media from the above IL-3 growth independent BaF3/MPLR1.1 cells were assayed for proliferative activity on BaF3/MPLR cells. Conditioned media from all nineteen IL-3 growth independent pools were found to have activity in the MTT proliferation assay (disclosed in Example VII). The positive media were reassayed for proliferative activity in the presence of 2  $\mu$ g/ml rat anti-mouse IL-3, or anti-mouse IL-4 or in the presence of both neutralizing antibodies (Pharmingen, San Diego, CA) to identify IL-3 growth independent mutants expressing those cytokines. (In a previous experiment, it was found that BaF3 cells also responded to IL-4.) Only conditioned medium from cells from plate #11 (designated "24-11" cells) was found to have activity that was not neutralized by IL-3 or IL-4 antibodies.

The mutagenesis and selection scheme described above was applied to five other BaF3/MPLR1 clones (BaF3/MPLR1 clones # 4, 9, 12, 15 and 18, designated as BaF3/MPLR1.4, .9, .12, .15 and .18, respectively). Seventeen isolates were found to have conditioned media which stimulated proliferation of BaF3/MPLR1 cells. Activity of all the media was found to be neutralized by anti-IL-3 or IL-4 antibodies alone or in combination. These clones were not characterized further.

The proliferative activity of conditioned media from the 24-11 pool was characterized in detail. The 24-11 pool was subdivided into nineteen subpools and conditioned media were retested for activity. All nineteen subpools (i.e. 24-11-1 thru 24-11-19) stimulated proliferation of IL-3 growth dependent BaF3/MPLR1 cells in the absence of exogenous IL-3. The activity was not

inhibited by IL-3 or IL-4 neutralizing antibodies or by a combination of both antibodies.

Two experiments were performed to determine the specificity of the 24-11 activity. The conditioned media were assayed for proliferative activity on control BaF3 cells that do not express the MPL receptor. In the absence of exogenous IL-3, proliferation of control BaF3 cells was not observed in the conditioned media from any of the nineteen 24-11 subpools. In a second experiment, proliferation activity was assayed for inhibition by purified soluble MPL receptor. BaF3/MPLR1 cells were cultured in GM media supplemented with 50% 24-11 conditioned media. To each sample was added Type I mouse soluble MPL receptor to a final concentration of 0.0, 0.625, 1.25, 2.5 or 5.0  $\mu\text{g/ml}$ . The result was scored 4 days later by MTT cell proliferation assay. The proliferative activity of the 24-11 conditioned media was completely blocked at 0.625 to 1.25  $\mu\text{g/ml}$  soluble MPL receptor. Soluble receptor concentrations that completely inhibited activity had no effect on IL-3 or IL-4 stimulation of BaF3/MPLR1 cells. The results indicated that soluble MPL receptor competed for the stimulatory activity of 24-11 media and is consistent with the hypothesis that 24-11 cells expressed the MPL receptor ligand.

Clones derived from 24-11 cells were isolated by plating at limiting dilutions. One clone, designated 24-11-5 #3, showed a high level of proliferative activity in its conditioned media relative to the 24-11 pool. The proliferative activity was found to be equal to a 1:2000 dilution of conditioned media from WEHI-3 cells (Becton Dickinson Labware).

#### Example VI. Construction of 24-11-5#3 cDNA library

Total RNA was prepared from  $2.7 \times 10^8$  24-11-5#3 cells using guanidine isothiocyanate followed by CsCl

centrifugation (Chirgwin et al., *ibid.*). Poly(A)<sup>+</sup> RNA was isolated using an OLIGOTEX-dT-mRNA isolation kit (Qiagen Inc., Chatsworth, CA) following the manufacturer's instructions.

5 First strand cDNA from 24-11-5#3 cells was synthesized in 4 separate parallel reactions. Each reaction contained 7  $\mu$ l of poly d(T)-selected poly(A)<sup>+</sup> 24-11-5#3 RNA at a concentration of 1.6  $\mu$ g/ $\mu$ l and 2.5  $\mu$ l of 20 pmole/ $\mu$ l first strand primer ZC6172 (SEQ ID NO: 14)  
10 containing an Xho I restriction site. The mixture was heated at 65°C for 4 minutes and cooled by chilling on ice. First strand cDNA synthesis was initiated by the addition of 8  $\mu$ l of first strand buffer (5x SUPERScript™ buffer; GIBCO BRL), 4  $\mu$ l of 100 mM dithiothreitol and 2  $\mu$ l  
15 of a deoxynucleotide triphosphate solution containing 10 mM each of dATP, dGTP, dTTP and 5-methyl-dCTP (Pharmacia LKB Biotechnology Inc.) to the RNA-primer mixture. The reaction mixture was incubated at 45° C for 4 minutes followed by the addition of 10  $\mu$ l of 200 U/ $\mu$ l RNase H<sup>-</sup>  
20 reverse transcriptase (GIBCO BRL). The efficiency of the first strand synthesis was analyzed in a parallel reaction by the addition of 10  $\mu$ Ci of <sup>32</sup>P- $\alpha$ dCTP to a 10  $\mu$ l aliquot from one of the reaction mixtures to label the reaction for analysis. The reactions were incubated at 45° C for 1  
25 hour followed by an incubation at 50° C for 15 minutes. Unincorporated <sup>32</sup>P- $\alpha$ dCTP in the labeled reaction was removed by chromatography on a 400 pore size gel filtration column (Clontech Laboratories). The unlabeled first strand reactions were pooled, and unincorporated  
30 nucleotides were removed by twice precipitating the cDNA in the presence of 32  $\mu$ g of glycogen carrier, 2.5 M ammonium acetate and 2.5 volume ethanol. The unlabeled cDNA was resuspended in 144  $\mu$ l water for use in second strand synthesis. The length of labeled first strand cDNA  
35 was determined by agarose gel electrophoresis.

Second strand synthesis was performed on the first strand cDNA under conditions that promoted first strand priming of second strand synthesis resulting in DNA hairpin formation. Three separate parallel second strand reactions were performed. Each second strand reaction contained 48  $\mu$ l of the unlabeled first strand cDNA, 16.5  $\mu$ l of water, 20  $\mu$ l of 5x polymerase I buffer (100 mM Tris: HCl, pH 7.4, 500 mM KCl, 25 mM MgCl<sub>2</sub>, 50 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>), 1  $\mu$ l of 100 mM dithiothreitol, 1  $\mu$ l of a solution containing 10 mM of each deoxynucleotide triphosphate, 3  $\mu$ l of 5 mM  $\beta$ -NAD, 1  $\mu$ l of 3 U/ $\mu$ l *E. coli* DNA ligase (New England Biolabs Inc.) and 5  $\mu$ l of 10 U/ $\mu$ l *E. coli* DNA polymerase I (Amersham Corp.). The reaction was assembled at room temperature and was incubated at room temperature for 5 minutes followed by the addition of 1.5  $\mu$ l of 2 U/ $\mu$ l RNase H (GIBCO BRL). A 10  $\mu$ l aliquot from one of the second strand synthesis reactions was labeled by the addition of 10  $\mu$ Ci <sup>32</sup>P- $\alpha$ dCTP to monitor the efficiency of second strand synthesis. The reactions were incubated at 15° C for two hours followed by a 15 minute incubation at room temperature. Unincorporated <sup>32</sup>P- $\alpha$ dCTP in the labeled reaction was removed by chromatography through a 400 pore size gel filtration column (Clontech Laboratories) before analysis by agarose gel electrophoresis. The unlabeled reactions were pooled and extracted with phenol/chloroform and chloroform followed by ethanol precipitation in the presence of 2.5 M ammonium acetate.

The single-stranded DNA of the hairpin structure was cleaved using mung bean nuclease. The reaction mixture contained 100  $\mu$ l of second strand cDNA, 20  $\mu$ l of 10x mung bean nuclease buffer (Stratagene Cloning Systems), 16  $\mu$ l of 100 mM dithiothreitol, 48  $\mu$ l of water, 10  $\mu$ l of mung bean nuclease dilution buffer (Stratagene Cloning Systems) and 6  $\mu$ l of 50 U/ $\mu$ l mung bean nuclease (Promega Corp.). The reaction was incubated at 37° C for 30 minutes. The reaction was terminated by the addition

of 20  $\mu$ l of 1 M Tris: HCl, pH 8.0 followed by sequential phenol/chloroform and chloroform extractions as described above. Following the extractions, the DNA was precipitated in ethanol and resuspended in water.

5           The resuspended cDNA was blunt-ended with T4 DNA polymerase. The cDNA, which was resuspended in 188  $\mu$ l of water, was mixed with 50  $\mu$ l 5x T4 DNA polymerase buffer (250 mM Tris:HCl, pH 8.0, 250 mM KCl, 25 mM  $MgCl_2$ ), 3  $\mu$ l 0.1 M dithiothreitol, 4  $\mu$ l of a solution containing 10 mM  
10 of each deoxynucleotide triphosphate and 5  $\mu$ l of 1 U/ $\mu$ l T4 DNA polymerase (Boehringer Mannheim Corp.). After an incubation of 30 minutes at 15° C, the reaction was terminated by the addition of 10  $\mu$ l of 0.5 M EDTA followed by serial phenol/chloroform and chloroform extractions as  
15 described above. The DNA was chromatographed through a 400 pore size gel filtration column (Clontech Laboratories Inc.) to remove trace levels of protein and to remove short cDNAs less than ~400 bp in length. The DNA was ethanol precipitated in the presence of 10  $\mu$ g glycogen  
20 carrier and 2.5 M ammonium acetate and was resuspended 15  $\mu$ l of water. Based on the incorporation of  $^{32}P$ - $\alpha$ dCTP, the yield of cDNA was estimated to be ~8  $\mu$ g from a starting mRNA template of 40  $\mu$ g.

*Eco* RI adapters were ligated onto the 5' ends of  
25 the cDNA described above to enable cloning into an expression vector. A 10  $\mu$ l aliquot of cDNA (~5  $\mu$ g) and 21  $\mu$ l of 65 pmole/ $\mu$ l of *Eco* RI adapter (Pharmacia LKB Biotechnology Inc.) were mixed with 4  $\mu$ l 10x ligase buffer (Promega Corp.), 3  $\mu$ l of 10 mM ATP and 3  $\mu$ l of 15 U/ $\mu$ l T4  
30 DNA ligase (Promega Corp.). The reaction was incubated overnight (~48 hours) at 9° C. The reaction was terminated by the addition of 140  $\mu$ l of water, 20  $\mu$ l of 10x H buffer (Boehringer Mannheim Corp.) and incubation at 65° C for 40 minutes. After incubation, the cDNA was extracted with  
35 phenol/chloroform and chloroform as described above and precipitated in the presence of 2.5 M ammonium acetate and



1.2 volume of isopropanol. Following centrifugation, the cDNA pellet was washed with 70% ethanol, air dried and resuspended in 89  $\mu$ l water.

To facilitate the directional cloning of the cDNA into an expression vector, the cDNA was digested with *Xho* I, resulting in a cDNA having a 5' *Eco* RI cohesive end and a 3' *Xho* I cohesive end. The *Xho* I restriction site at the 3' end of the cDNA had been previously introduced using the ZC6172 primer (SEQ ID NO: 14). Restriction enzyme digestion was carried out in a reaction mixture containing 89  $\mu$ l of cDNA described above, 10  $\mu$ l of 10x H buffer (Promega Corp.) and 1.5  $\mu$ l of 40 U/ $\mu$ l *Xho* I (Boehringer Mannheim Corp.). Digestion was carried out at 37° C for 1 hour. The reaction was terminated by serial phenol/chloroform and chloroform extractions and chromatography through a 400 pore size gel filtration column (Clontech Laboratories Inc.).

The cDNA was ethanol precipitated, washed with 70% ethanol, air dried and resuspended in 20  $\mu$ l of 1x gel loading buffer (10 mM Tris:HCl, pH 8.0, 1 mM EDTA, 5% glycerol and 0.125% bromphenol blue). The resuspended cDNA was heated to 65° C for 5 minutes, cooled on ice and electrophoresed on a 0.8% low melt agarose gel (SEA PLAQUE GTG™ low melt agarose; FMC Corp.). The contaminating adapters and cDNA below 0.5 Kb in length were excised from the gel. The electrodes were reversed, and the cDNA was electrophoresed until concentrated near the lane origin. The area of the gel containing the concentrated cDNA was excised and placed in a microfuge tube, and the approximate volume of the gel slice was determined. An aliquot of water approximately three times the volume of the gel slice (300  $\mu$ l) was added to the tube, and the agarose was melted by heating to 65° C for 15 minutes. Following equilibration of the sample to 45° C, 5  $\mu$ l of 1 U/ $\mu$ l  $\beta$ -agarase I (New England Biolabs, Inc.) was added, and the mixture was incubated for 90 minutes at 45° C to

digest the agarose. After incubation, 40  $\mu$ l of 3 M Na acetate was added to the sample, and the mixture was incubated on ice for 15 minutes. The sample was centrifuged at 14,000 x g for 15 minutes at room temperature to remove undigested agarose followed by chromatography through a 400 pore size gel filtration column (Clontech Laboratories). The cDNA was ethanol precipitated, washed in 70% ethanol, air-dried and resuspended in 70  $\mu$ l water for the kinase reaction to phosphorylate the ligated *Eco* RI adapters.

To the 70  $\mu$ l cDNA solution was added 10  $\mu$ l 10x ligase buffer (Stratagene Cloning Systems), and the mixture was heated to 65° C for 5 minutes. The mixture was cooled on ice, and 16  $\mu$ l 10 mM ATP and 4  $\mu$ l of 10 U/ $\mu$ l T4 polynucleotide kinase (Stratagene Cloning Systems) were added. The reaction mixture was incubated at 37° C for 1 hour and was terminated by heating to 65° C for 10 minutes followed by serial extractions with phenol/chloroform and chloroform. The phosphorylated cDNA was ethanol precipitated in the presence of 2.5 M ammonium acetate, washed with 70% ethanol, air dried and resuspended in 10  $\mu$ l of water. The concentration of the phosphorylated cDNA was estimated to be ~40 fmole/ $\mu$ l.

The pDX mammalian expression vector (disclosed in U.S. Patent No. 4,959,318) (Figure 2) was modified to accept 24-11-5#3 cDNA that had been synthesized with *Eco* RI-Xho I ends. An endogeneous *Sal* I site on pDX was eliminated by digesting the plasmid with *Sal* I and recircularizing the plasmid following blunting of the *Sal* I cohesive ends with T4 DNA polymerase. The recircularized plasmid was digested with *Eco* RI and to it was ligated a short polylinker sequence consisting of two complementary oligonucleotides, ZC6936 (SEQ ID NO: 15) and ZC6937 (SEQ ID NO: 16), to yield plasmid pDX.ES. The introduced polylinker sequence on pDX.ES contained *Eco* RI

and *Sal* I sites to facilitate directional cloning of 24-11-5 cDNA synthesized with *Eco* RI-*Xho* I ends.

A plasmid cDNA library was prepared by ligating *Eco* RI-*Xho* I 24-11-5 cDNA into *Eco* RI/*Sal* I digested pDX.ES. The ligation mixture was electroporated into *E. coli* (ELECTROMAX DH10B™ competent cells; GIBCO BRL, Gaithersburg, MD) using a gene pulser/pulse controller and 0.2 cm cuvette (Bio-Rad Laboratories, Hercules, CA) employing a 0.2 KV, 400 ohm and 25  $\mu$ FAD. The cells were diluted to 1.5 ml in Luria broth and incubated at 37°C for 45 minutes followed by the addition of 0.75 ml of 50% glycerol. The transfected cells were aliquotted and stored at -70°C until use. Eighty fmoles of cDNA gave rise to over 700,000 independent recombinant plasmids.

#### Example VII. Expression Screening of 24-11-5 cDNA Library for MPL Activity

The 24-11-5#3 cDNA library was plated onto approximately two thousand 10 cm diameter Luria broth agar plates supplemented with 100  $\mu$ g/ml ampicillin. The plating density was between 200 to 250 bacterial colonies per plate. Plasmid DNA for transfection into BHK 570 cells was prepared from each bacterial plate using Magic minipreps DNA purification resin (Promega Corp.), according to the manufacturer's instruction. Plasmid DNAs were stored at -20° C until transfection into BHK 570 cells.

Plasmid pools of 24-11-5#3 cDNA, each containing approximately 200 to 250 cDNA clones were transfected into BHK 570 cells using LIPOFECTAMINE™ (GIBCO BRL). Twenty  $\mu$ l of 30 ng/ $\mu$ l DNA was added to 20  $\mu$ l of a 1:10 dilution of LIPOFECTAMINE™ solution and incubated at room temperature for 30 minutes. Following the incubation, 160  $\mu$ l of serum-free media (Hams F12: Dulbeccos MEM (1:1) supplemented with 2 mM L-glutamine, 0.11 mg/ml sodium pyruvate, 5  $\mu$ g/ml insulin, 5  $\mu$ g/ml fetuin, 10  $\mu$ g/ml transferrin, 2 ng/ml

selenium IV oxide and 25 mM HEPES buffer) were added to the DNA/LIPOFECTAMINE™ mixture and transferred to a 24 well microtiter plate containing ~100,000 BHK 570 cells. The cells were incubated at 37° C under 5% CO<sub>2</sub> for 4 hours, after which was added 200 ml of BHK Growth Media (Dulbecco's modified Eagles's media supplemented with 2 mM L-glutamine, 0.11 mg/ml sodium pyruvate, 5% heat inactivated fetal calf serum and 100x PSN antibiotics (GIBCO BRL)). The cells were incubated for 16 hours. The media was removed and replaced with 0.5 ml of fresh BHK Growth Media, which was conditioned for 48 hours before being assayed for MPL activity.

A cell proliferation assay was used to detect the presence of MPL activity in conditioned media of library transfected BHK 570 cells. One hundred µl of conditioned media was added to 100 µl of 10<sup>5</sup>/ml washed BaF3/MPLR1.1 cells in RPMI 1640 media (JRH Bioscience Inc., Lenexa, KS) supplemented with 2 mM L-glutamine, PSN antibiotics (GIBCO BRL), 0.00036% 2 mercaptoethanol and 10% heat inactivated fetal calf serum. The assay cells were incubated for 3 days at 37° C under 5% CO<sub>2</sub> before assaying for proliferation.

Cell proliferation in the presence of MPL was quantified using a colorimetric assay based on the metabolic breakdown of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (Mosman, J. Immunol. Meth. 65: 55-63, 1983). Twenty µl of a 10 mg/ml solution of MTT (Polyscience, Inc., Warrington, PA) was added to 100 µl of BaF3/MPLR1.1 assay cells, and the cells were incubated at 37° C. After 4 hours, 200 µl of 0.04 N HCl in isopropanol was added, the solution was mixed, and the adsorbance of the sample was read at 570 nm on a model EL320 ELISA reader (Bio-Tek Instruments Inc., Highland Park, VT).

One plasmid pool found to be positive, designated T1081, was transfected into BHK 570 cells.

Supernatant from the transfectants gave a positive signal in the MTT proliferation assay. PCR and antibody neutralization experiments demonstrated that the activity was not due to IL-3 or IL-4.

5           Plasmids from the positive pool were used to transform *E. coli* DH10B, and cells were plated (42 plates with approximately 15-20 colonies per plate, 10 plates with approximately 90 colonies per plate and 8 plates with approximately 250 colonies per plate). A replica of each  
10 plate was made and stored at 4°C. The colonies on the original plates were scraped and allowed to outgrow in liquid culture for several more hours, then DNA was prepared.

          The plasmid DNA from the sub-pools was  
15 transfected into BHK 570 cells, and cell supernatants were collected and assayed as above. After approximately two hours, one sub-pool (#22) was scored as positive by microscopic examination (elongated cell shape). Several hours later two additional sub-pools (#19 and #28) were  
20 also scored positive. Remaining supernatants from each positive sub-pool were assayed against the control BaF3 cells and found to have no activity. In addition, the activity from the three positive sub-pools was found to be inhibited by the soluble Type I MPL receptor.

25           The replica plates from the three positive sub-pools were allowed to grow for several hours, then individual colonies were picked and used to inoculate 3-ml cultures. The cultures were grown approximately 8 hours at 37°C, then DNA was prepared by the miniprep method  
30 as described above. Plasmid DNA was transfected into BHK 570 cells, and supernatants were harvested approximately 10 hours later and assayed for activity. After one hour, one clone (designated T1081-19-215, corresponding to sub-pool #19) was scored positive. This clone was restreaked  
35 for single colonies. DNA was prepared from twelve colonies and transfected into BHK 570 cells. All twelve

transfectants were later scored positive in the assay. DNA from one of the twelve positive colonies was transformed into *E. coli* DH5 $\alpha$ . The plasmid was designated pZGmpl-1081. This transformant has been deposited with American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD under accession number 69566.

The nucleotide sequence of the cDNA encoding the hematopoietic protein was determined (SEQ ID NO: 1). Analysis of the encoded amino acid sequence (SEQ ID NO: 2) indicated that the amino terminus of the mature protein is at amino acid residue 45. Two methionine codons, at positions 105 and 174 of SEQ ID NO: 1, appear to be initiation codons, with the major site of initiation expected to be at position 174.

#### Example VIII. Hematopoietic Activity of Recombinant Protein

Marrow was harvested from femurs and tibias of a female CD-1 post-pregnant mouse into 25 ml of CATCH buffer (99 mg theophylline, 0.75 g sodium citrate, 75 mg adenosine, 20ml Hank's balanced saline solution Ca<sup>++</sup> Mg<sup>++</sup>-free, per 200 ml in dH<sub>2</sub>O; pH 7.4). Cells were suspended into single cell suspension by pipeting with a 25 ml pipet. The volume was brought up to 50 ml with CATCH buffer, and the cells were pelleted at 1000 rpm for 7 minutes. The pellet was resuspended in 25 ml CATCH buffer and incubated in a T75 tissue culture flask for a first round of plastic adherence at 37°C for 2 hours. Non-adherent cells were harvested by the addition of 15 ml of alpha-MEM + 10% FBS (+L-glutamine, NaPyruvate, PSN) and washing non-adherent cells into suspension. The media and non-adherent cells were transferred into a 50 ml tube and centrifuged at 1000 rpm for 7 minutes to pellet cells. The pellet was resuspended in 25 ml CATCH buffer and incubated in a T75 flask for a second round of plastic adherence as described above for the first round.

Following the final centrifugation and resuspension, the cells were counted. One-half ml of cells at 576,000 cells/ml was plated into 24-well tissue culture plates, together with sample media from control BHK cells or with  
5 conditioned media from BHK cells transfected with pZGmpl-1081. After three days incubation at 37°C, the cells were harvested and stained as described below.

One hundred fifty µl of cells were harvested from the control well treated with standard conditioned medium.  
10 50 µl of cells were harvested from the well treated with conditioned medium from BHK cells transfected with pZGmpl-1081. These samples were cytopspun and standard microscope slides were prepared.

The cytopspin slides were fixed in 100% methanol.  
15 The slides were flooded with 1:1 Wright's (0.5 g Wright stain in 300 ml methanol)/H<sub>2</sub>O for 6 minutes, washed with water, and dried. Slides were then flooded with Geimsa stain (Sigma Chemical Corp.) in Sorensen buffer (2.28 g KH<sub>2</sub>PO<sub>4</sub>/2.38g NaPO<sub>4</sub> in 250 ml H<sub>2</sub>O), washed with water, and  
20 dried.

After adjusting for the volumes used, the medium sample contained 120 megakaryocytes per 150 µl volume as compared to 9 megakaryocytes per 150 µl volume of control medium. In addition, these megakaryocytes in the treated  
25 experimental sample were observed microscopically to be significantly larger in size than control cells and to have significantly higher staining for polynuclei content.

Conditioned media from the mutant BaF3/MPLR1.1 line 24-11-5 #3 was collected in the absence of serum and concentrated 20-fold on a 10Kd cut-off Amicon Inc.  
30 (Beverly, MA) filtration device. Marrow was harvested from mouse femurs and suspended in Iscove's Modified Dulbecco's medium + 15% FCS. Following suspension, nucleated cells were counted and plated at 75,000 cells/ml  
35 with 0.9 ml/plate in semi-solid medium containing 50% methylcellulose, 15% FCS, 10% BSA, and 0.6% PSN in 1 ml

tissue culture plates. Various conditioned medium and control samples were added to bring the total volume to 1 ml. Plates were incubated at 37°C/5% CO<sub>2</sub> for 6 days and then examined microscopically for counts of Granulocyte/Macrophage (GM) colonies. Plates incubated in the presence of the 24-11-5 #3 conditioned medium were observed to have weak GMCSF-like activity, producing a colony count of 25, compared with a count of zero for the negative control sample, and a count of 130 for a plate stimulated with a positive control (pokeweed mitogen spleen concentrated medium + erythropoietin).

From the foregoing, it will be appreciated that, although specific embodiments of the invention have been described herein for purposes of illustration, various modifications may be made without deviating from the spirit and scope of the invention. Accordingly, the invention is not limited except as by the appended claims.



## SEQUENCE LISTING

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(B) STREET: 1201 Eastlake Avenue East  
(C) CITY: Seattle  
(D) STATE: WA  
(E) COUNTRY: USA  
(F) ZIP: 98102

## (v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk  
(B) COMPUTER: IBM PC compatible  
(C) OPERATING SYSTEM: PC-DOS/MS-DOS  
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

## (vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER:  
(B) FILING DATE:  
(C) CLASSIFICATION:

## (viii) ATTORNEY/AGENT INFORMATION:

(A) NAME: Parker, Gary E  
(B) REGISTRATION NUMBER: 31-648  
(C) REFERENCE/DOCKET NUMBER: 94-2PC

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(A) TELEPHONE: 206-442-6673

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## (2) INFORMATION FOR SEQ ID NO:1:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1486 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: double

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

## (vii) IMMEDIATE SOURCE:

(B) CLONE: 1081

## (ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION: 105..1241

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

CCTCGTGCCG GTCCTGAGGC CCTTCTCCAC CCGGACAGAG TCCTTGCCCC ACCTCTCTCC 60

CACCCGACTC TGCCGAAAGA AGCACAGAAG CTCAAGCCGC CTCC ATG GCC CCA GGA 116  
Met Ala Pro Gly  
1AAG ATT CAG GGG AGA GGC CCC ATA CAG GGA GCC ACT TCA GTT AGA CAC 164  
Lys Ile Gln Gly Arg Gly Pro Ile Gln Gly Ala Thr Ser Val Arg His  
5 10 15 20CTG GCC AGA ATG GAG CTG ACT GAT TTG CTC CTG GCG GCC ATG CTT CTT 212  
Leu Ala Arg Met Glu Leu Thr Asp Leu Leu Leu Ala Ala Met Leu Leu  
25 30 35

GCA GTG GCA AGA CTA ACT CTG TCC AGC CCC GTA GCT CCT GCC TGT GAC	260
Ala Val Ala Arg Leu Thr Leu Ser Ser Pro Val Ala Pro Ala Cys Asp	
40 45 50	
CCC AGA CTC CTA AAT AAA CTG CTG CGT GAC TCC CAC CTC CTT CAC AGC	308
Pro Arg Leu Leu Asn Lys Leu Leu Arg Asp Ser His Leu Leu His Ser	
55 60 65	
CGA CTG AGT CAG TGT CCC GAC GTC GAC CCT TTG TCT ATC CCT GTT CTG	356
Arg Leu Ser Gln Cys Pro Asp Val Asp Pro Leu Ser Ile Pro Val Leu	
70 75 80	
CTG CCT GCT GTG GAC TTT AGC CTG GGA GAA TGG AAA ACC CAG ACG GAA	404
Leu Pro Ala Val Asp Phe Ser Leu Gly Glu Trp Lys Thr Gln Thr Glu	
85 90 95 100	
CAG AGC AAG GCA CAG GAC ATT CTA GGG GCA GTG TCC CTT CTA CTG GAG	452
Gln Ser Lys Ala Gln Asp Ile Leu Gly Ala Val Ser Leu Leu Leu Glu	
105 110 115	
GGA GTG ATG GCA GCA CGA GGA CAG TTG GAA CCC TCC TGC CTC TCA TCC	500
Gly Val Met Ala Ala Arg Gly Gln Leu Glu Pro Ser Cys Leu Ser Ser	
120 125 130	
CTC CTG GGA CAG CTT TCT GGG CAG GTT CGC CTC CTC TTG GGG GCC CTG	548
Leu Leu Gly Gln Leu Ser Gly Gln Val Arg Leu Leu Leu Gly Ala Leu	
135 140 145	
CAG GGC CTC CTA GGA ACC CAG CTT CCT CTA CAG GGC AGG ACC ACA GCT	596
Gln Gly Leu Leu Gly Thr Gln Leu Pro Leu Gln Gly Arg Thr Thr Ala	
150 155 160	
CAC AAG GAC CCC AAT GCC CTC TTC TTG AGC TTG CAA CAA CTG CTT CGG	644
His Lys Asp Pro Asn Ala Leu Phe Leu Ser Leu Gln Gln Leu Leu Arg	
165 170 175 180	

GGA AAG GTG CGC TTC CTG CTT CTG GTA GAA GGT CCC ACC CTC TGT GTC	692
Gly Lys Val Arg Phe Leu Leu Leu Val Glu Gly Pro Thr Leu Cys Val	
185 190 195	
AGA CGG ACC CTG CCA ACC ACA GCT GTC CCA AGC AGT ACT TCT CAA CTC	740
Arg Arg Thr Leu Pro Thr Thr Ala Val Pro Ser Ser Thr Ser Gln Leu	
200 205 210	
CTC ACA CTA AAC AAG TTC CCA AAC AGG ACT TCT GGA TTG TTG GAG ACG	788
Leu Thr Leu Asn Lys Phe Pro Asn Arg Thr Ser Gly Leu Leu Glu Thr	
215 220 225	
AAC TTC AGT GTC ACA GCC AGA ACT GCT GGC CCT GGA CTT CTG AGC AGG	836
Asn Phe Ser Val Thr Ala Arg Thr Ala Gly Pro Gly Leu Leu Ser Arg	
230 235 240	
CTT CAG GGA TTC AGA GTC AAG ATT ACT CCT GGT CAG CTA AAT CAA ACC	884
Leu Gln Gly Phe Arg Val Lys Ile Thr Pro Gly Gln Leu Asn Gln Thr	
245 250 255 260	
TCC AGG TCC CCA GTC CAA ATC TCT GGA TAC CTG AAC AGG ACA CAC GGA	932
Ser Arg Ser Pro Val Gln Ile Ser Gly Tyr Leu Asn Arg Thr His Gly	
265 270 275	
CCT GTG AAT GGA ACT CAT GGG CTC TTT GCT GGA ACC TCA CTT CAG ACC	980
Pro Val Asn Gly Thr His Gly Leu Phe Ala Gly Thr Ser Leu Gln Thr	
280 285 290	
CTG GAA GCC TCA GAC ATC TCG CCC GGA GCT TTC AAC AAA GGC TCC CTG	1028
Leu Glu Ala Ser Asp Ile Ser Pro Gly Ala Phe Asn Lys Gly Ser Leu	
295 300 305	
GCA TTC AAC CTC CAG GGT GGA CTT CCT CCT TCT CCA AGC CTT GCT CCT	1076
Ala Phe Asn Leu Gln Gly Gly Leu Pro Pro Ser Pro Ser Leu Ala Pro	
310 315 320	
GAT GGA CAC ACA CCC TTC CCT CCT TCA CCT GCC TTG CCC ACC ACC CAT	1124
Asp Gly His Thr Pro Phe Pro Pro Ser Pro Ala Leu Pro Thr Thr His	
325 330 335 340	

GGA TCT CCA CCC CAG CTC CAC CCC CTG TTT CCT GAC CCT TCC ACC ACC 1172  
 Gly Ser Pro Pro Gln Leu His Pro Leu Phe Pro Asp Pro Ser Thr Thr  
                   345                  350                  355

ATG CCT AAC TCT ACC GCC CCT CAT CCA GTC ACA ATG TAC CCT CAT CCC 1220  
 Met Pro Asn Ser Thr Ala Pro His Pro Val Thr Met Tyr Pro His Pro  
                   360                  365                  370

AGG AAT TTG TCT CAG GAA ACA TAGCGCGGGC ACTGGCCCAG TGAGCGTCTG 1271  
 Arg Asn Leu Ser Gln Glu Thr  
                   375

CAGCTTCTCT CGGGGACAAG CTTCCCCAGG AAGGCTGAGA GGCAGCTGCA TCTGCTCCAG 1331

ATGTTCTGCT TTCACCTAAA AGGCCCTGGG GAAGGGATAC ACAGCACTGG AGATTGTAAA 1391

ATTTTAGGAG CTATTTTTTT TTAACCTATC AGCAATATTC ATCAGAGCAG CTAGCGATCT 1451

TTGGTCTATT TTCGGTATAA ATTTGAAAAT CACTA 1486

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 379 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met Ala Pro Gly Lys Ile Gln Gly Arg Gly Pro Ile Gln Gly Ala Thr  
   1                  5                  10                  15

Ser Val Arg His Leu Ala Arg Met Glu Leu Thr Asp Leu Leu Leu Ala  
                   20                  25                  30

Ala Met Leu Leu Ala Val Ala Arg Leu Thr Leu Ser Ser Pro Val Ala  
 35 40 45

Pro Ala Cys Asp Pro Arg Leu Leu Asn Lys Leu Leu Arg Asp Ser His  
 50 55 60

Leu Leu His Ser Arg Leu Ser Gln Cys Pro Asp Val Asp Pro Leu Ser  
 65 70 75 80

Ile Pro Val Leu Leu Pro Ala Val Asp Phe Ser Leu Gly Glu Trp Lys  
 85 90 95

Thr Gln Thr Glu Gln Ser Lys Ala Gln Asp Ile Leu Gly Ala Val Ser  
 100 105 110

Leu Leu Leu Glu Gly Val Met Ala Ala Arg Gly Gln Leu Glu Pro Ser  
 115 120 125

Cys Leu Ser Ser Leu Leu Gly Gln Leu Ser Gly Gln Val Arg Leu Leu  
 130 135 140

Leu Gly Ala Leu Gln Gly Leu Leu Gly Thr Gln Leu Pro Leu Gln Gly  
 145 150 155 160

Arg Thr Thr Ala His Lys Asp Pro Asn Ala Leu Phe Leu Ser Leu Gln  
 165 170 175

Gln Leu Leu Arg Gly Lys Val Arg Phe Leu Leu Leu Val Glu Gly Pro  
 180 185 190

Thr Leu Cys Val Arg Arg Thr Leu Pro Thr Thr Ala Val Pro Ser Ser  
 195 200 205

Thr Ser Gln Leu Leu Thr Leu Asn Lys Phe Pro Asn Arg Thr Ser Gly  
 210 215 220

Leu Leu Glu Thr Asn Phe Ser Val Thr Ala Arg Thr Ala Gly Pro Gly  
 225 230 235 240

Leu Leu Ser Arg Leu Gln Gly Phe Arg Val Lys Ile Thr Pro Gly Gln  
                     245                    250                    255  
 Leu Asn Gln Thr Ser Arg Ser Pro Val Gln Ile Ser Gly Tyr Leu Asn  
                     260                    265                    270  
 Arg Thr His Gly Pro Val Asn Gly Thr His Gly Leu Phe Ala Gly Thr  
                     275                    280                    285  
 Ser Leu Gln Thr Leu Glu Ala Ser Asp Ile Ser Pro Gly Ala Phe Asn  
                     290                    295                    300  
 Lys Gly Ser Leu Ala Phe Asn Leu Gln Gly Gly Leu Pro Pro Ser Pro  
 305                    310                    315                    320  
 Ser Leu Ala Pro Asp Gly His Thr Pro Phe Pro Pro Ser Pro Ala Leu  
                     325                    330                    335  
 Pro Thr Thr His Gly Ser Pro Pro Gln Leu His Pro Leu Phe Pro Asp  
                     340                    345                    350  
 Pro Ser Thr Thr Met Pro Asn Ser Thr Ala Pro His Pro Val Thr Met  
                     355                    360                    365  
 Tyr Pro His Pro Arg Asn Leu Ser Gln Glu Thr  
                     370                    375

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 42 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(vii) IMMEDIATE SOURCE:

- (B) CLONE: ZC5499

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

CGAGCCACTT TCTGCACTCC TCGAGTTTTT TTTTTTTTTT TT

42

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 45 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(vii) IMMEDIATE SOURCE:

(B) CLONE: ZC5746

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

GAGAGAGAGA GAGAATTCAT GCCCTCCTGG GCCCTCTTCA TGGTC

45

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 52 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(vii) IMMEDIATE SOURCE:

(B) CLONE: ZC5762

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

AGAGAGAGAG AGAGCTCGAG TCAAGGCTGC TGCCAATAGC TTAGTGGTAG GT

52



## (2) INFORMATION FOR SEQ ID NO:6:

- (i) SEQUENCE CHARACTERISTICS:  
    (A) LENGTH: 30 base pairs  
    (B) TYPE: nucleic acid  
    (C) STRANDEDNESS: single  
    (D) TOPOLOGY: linear

- (vii) IMMEDIATE SOURCE:  
    (B) CLONE: ZC5742

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

GACCCTGGAG CTGCGCCCGC GATCTCGCTA

30

## (2) INFORMATION FOR SEQ ID NO:7:

- (i) SEQUENCE CHARACTERISTICS:  
    (A) LENGTH: 49 base pairs  
    (B) TYPE: nucleic acid  
    (C) STRANDEDNESS: single  
    (D) TOPOLOGY: linear

- (vii) IMMEDIATE SOURCE:  
    (B) CLONE: ZC6091

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

GAGCACAGAA TTCACTACTC GAGGCGGCCG CTTTTTTTTT TTTTTTTTTT

49

## (2) INFORMATION FOR SEQ ID NO:8:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 45 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (vii) IMMEDIATE SOURCE:

- (B) CLONE: ZC6603

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

GAGGAATTCG CAGAAGCCAT GCCCTCTTGG GCCCTCTTCA TGGTC

45

## (2) INFORMATION FOR SEQ ID NO:9:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 8 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

Val Arg Thr Ser Pro Ala Gly Glu  
1 5

## (2) INFORMATION FOR SEQ ID NO:10:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 48 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(vii) IMMEDIATE SOURCE:  
(B) CLONE: ZC6704

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

GAAGAGGAAT TCACCATGGA TGTCTTCTTG CTGGCCTTGG GCACAGAG

48

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 60 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(vii) IMMEDIATE SOURCE:  
(B) CLONE: ZC6703

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

CGACTTTACC TCGAGTGCTA CTGATGCTCT TCTGCCAGCA GTCTCGGAGC CCGTGGACAC

60

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 42 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(vii) IMMEDIATE SOURCE:  
(B) CLONE: ZC6707

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

AATTCGCCAT GGGACTCGAG CATCACCATC ACCATCACTG AG

42

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 42 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(vii) IMMEDIATE SOURCE:

(B) CLONE: ZC6706

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

GATCCTCAGT GATGGTGATG GTGATGCTCG AGTCCCATGG CG

42

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 47 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(vii) IMMEDIATE SOURCE:

(B) CLONE: ZC6172

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

GTCGGTGCTC AGCATTCAC TACTCGAGGGT TTTTTTTTTT TTTTTT

47

## (2) INFORMATION FOR SEQ ID NO:15:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 28 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (vii) IMMEDIATE SOURCE:

- (B) CLONE: ZC6936

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

AATTGGCGGC CGCGTCGACT CGTGGATG

28

## (2) INFORMATION FOR SEQ ID NO:16:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 28 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (vii) IMMEDIATE SOURCE:

- (B) CLONE: ZC6937

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

AATTCATCCA CGAGTCGACG CGGCCGCC

28

## (2) INFORMATION FOR SEQ ID NO:17:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 633 amino acids
- (B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

Met	Pro	Ser	Trp	Ala	Leu	Phe	Met	Val	Thr	Ser	Cys	Leu	Leu	Leu	Ala
1				5					10					15	
Leu	Pro	Asn	Gln	Ala	Gln	Val	Thr	Ser	Gln	Asp	Val	Phe	Leu	Leu	Ala
			20						25					30	
Leu	Gly	Thr	Glu	Pro	Leu	Asn	Cys	Phe	Ser	Gln	Thr	Phe	Glu	Asp	Leu
			35					40					45		
Thr	Cys	Phe	Trp	Asp	Glu	Glu	Glu	Ala	Ala	Pro	Ser	Gly	Thr	Tyr	Gln
			50					55					60		
Leu	Leu	Tyr	Ala	Tyr	Arg	Gly	Glu	Lys	Pro	Arg	Ala	Cys	Pro	Leu	Tyr
					70					75					80
Ser	Gln	Ser	Val	Pro	Thr	Phe	Gly	Thr	Arg	Tyr	Val	Cys	Gln	Phe	Pro
					85					90				95	
Ala	Gln	Asp	Glu	Val	Arg	Leu	Phe	Phe	Pro	Leu	His	Leu	Trp	Val	Lys
			100						105					110	
Asn	Val	Ser	Leu	Asn	Gln	Thr	Leu	Ile	Gln	Arg	Val	Leu	Phe	Val	Asp
			115					120					125		
Ser	Val	Gly	Leu	Pro	Ala	Pro	Pro	Arg	Val	Ile	Lys	Ala	Arg	Gly	Gly
			130					135					140		
Ser	Gln	Pro	Gly	Glu	Leu	Gln	Ile	His	Trp	Glu	Ala	Pro	Ala	Pro	Glu
					150					155					160

Ile Ser Asp Phe Leu Arg His Glu Leu Arg Tyr Gly Pro Thr Asp Ser  
 165 170 175  
 Ser Asn Ala Thr Ala Pro Ser Val Ile Gln Leu Leu Ser Thr Glu Thr  
 180 185 190  
 Cys Cys Pro Thr Leu Trp Met Pro Asn Pro Val Pro Val Leu Asp Gln  
 195 200 205  
 Pro Pro Cys Val His Pro Thr Ala Ser Gln Pro His Gly Pro Val Arg  
 210 215 220  
 Thr Ser Pro Ala Gly Glu Ala Pro Phe Leu Thr Val Lys Gly Gly Ser  
 225 230 235 240  
 Cys Leu Val Ser Gly Leu Gln Ala Gly Lys Ser Tyr Trp Leu Gln Leu  
 245 250 255  
 Arg Ser Gln Pro Asp Gly Val Ser Leu Arg Gly Ser Trp Gly Pro Trp  
 260 265 270  
 Ser Phe Pro Val Thr Val Asp Leu Pro Gly Asp Ala Val Thr Ile Gly  
 275 280 285  
 Leu Gln Cys Phe Thr Leu Asp Leu Lys Met Val Thr Cys Gln Trp Gln  
 290 295 300  
 Gln Gln Asp Arg Thr Ser Ser Gln Gly Phe Phe Arg His Ser Arg Thr  
 305 310 315 320  
 Arg Cys Cys Pro Thr Asp Arg Asp Pro Thr Trp Glu Lys Cys Glu Glu  
 325 330 335  
 Glu Glu Pro Arg Pro Gly Ser Gln Pro Ala Leu Val Ser Arg Cys His  
 340 345 350  
 Phe Lys Ser Arg Asn Asp Ser Val Ile His Ile Leu Val Glu Val Thr  
 355 360 365

Thr Ala Gln Gly Ala Val His Ser Tyr Leu Gly Ser Pro Phe Trp Ile  
 370 375 380  
 His Gln Ala Val Leu Leu Pro Thr Pro Ser Leu His Trp Arg Glu Val  
 385 390 395 400  
 Ser Ser Gly Arg Leu Glu Leu Glu Trp Gln His Gln Ser Ser Trp Ala  
 405 410 415  
 Ala Gln Glu Thr Cys Tyr Gln Leu Arg Tyr Thr Gly Glu Gly Arg Glu  
 420 425 430  
 Asp Trp Lys Val Leu Glu Pro Ser Leu Gly Ala Arg Gly Gly Thr Leu  
 435 440 445  
 Glu Leu Arg Pro Arg Ala Arg Tyr Ser Leu Gln Leu Arg Ala Arg Leu  
 450 455 460  
 Asn Gly Pro Thr Tyr Gln Gly Pro Trp Ser Ala Trp Ser Pro Pro Ala  
 465 470 475 480  
 Arg Val Ser Thr Gly Ser Glu Thr Ala Trp Ile Thr Leu Val Thr Ala  
 485 490 495  
 Leu Leu Leu Val Leu Ser Leu Ser Ala Leu Leu Gly Leu Leu Leu Leu  
 500 505 510  
 Lys Trp Gln Phe Pro Ala His Tyr Arg Arg Leu Arg His Ala Leu Trp  
 515 520 525  
 Pro Ser Leu Pro Asp Leu His Arg Val Leu Gly Gln Tyr Leu Arg Asp  
 530 535 540  
 Thr Ala Ala Leu Ser Pro Ser Lys Ala Thr Val Thr Asp Ser Cys Glu  
 545 550 555 560  
 Glu Val Glu Pro Ser Leu Leu Glu Ile Leu Pro Lys Ser Ser Glu Ser  
 565 570 575



Thr Pro Leu Pro Leu Cys Pro Ser Gln Pro Gln Met Asp Tyr Arg Gly  
580 585 590

Leu Gln Pro Cys Leu Arg Thr Met Pro Leu Ser Val Cys Pro Pro Met  
595 600 605

Ala Glu Thr Gly Ser Cys Cys Thr Thr His Ile Ala Asn His Ser Tyr  
610 615 620

Leu Pro Leu Ser Tyr Trp Gln Gln Pro  
625 630

Claims

What is claimed is:

1. A method for obtaining cells that produce a ligand for an orphan receptor comprising:

(a) providing parent cells, wherein growth of said parent cells is dependent upon an exogenous growth factor;

(b) transfecting said parent cells with a DNA construct encoding an orphan receptor to produce transfected cells expressing said orphan receptor encoded by said DNA construct;

(c) exposing said transfected cells to mutagenizing conditions to produce mutagenized cells;

(d) culturing said mutagenized cells under conditions in which cell survival is dependent upon autocrine growth factor production;

(e) recovering progeny cells that survive said culturing step; and

(f) screening said progeny cells to identify cells that produce a ligand for said orphan receptor.

2. A method according to claim 1 wherein said screening step comprises assaying media conditioned by said progeny cells for growth-promoting activity on said transfected cells.

3. A method according to claim 2 wherein said screening step further comprises assaying media conditioned by said progeny cells for growth-promoting activity on said parent cells.

4. A method according to claim 1 wherein said screening step comprises culturing said progeny cells in the presence of an antibody to a known growth factor.

5. A method according to claim 1 wherein said screening step comprises assaying media conditioned by said progeny cells for growth-promoting activity on said transfected cells in the presence of an antibody to a known growth factor.

6. A method according to claim 1 wherein said screening step comprises culturing said progeny cells in the presence of a soluble form of said orphan receptor.

7. A method according to claim 1 wherein said screening step comprises assaying media conditioned by said progeny cells for growth-promoting activity on said transfected cells in the presence of a soluble form of said orphan receptor.

8. A method according to claim 1 wherein, prior to said culturing step, said mutagenized cells are cultured in the presence of said exogenous growth factor.

9. A method according to claim 1 wherein the parent cells do not express detectable levels of the orphan receptor.

10. A method according to claim 1 wherein said mutagenizing step comprises chemical treatment.

11. A method according to claim 1 wherein said parent cells are myeloid or lymphoid progenitor cells.

12. A method for preparing a polynucleotide molecule that encodes a ligand for an orphan receptor comprising:

(a) providing parent cells, wherein growth of said cells is dependent upon an exogenous growth factor;

(b) transfecting said parent cells with a DNA construct encoding an orphan receptor to produce transfected cells expressing said orphan receptor encoded by said DNA construct;

(c) exposing said transfected cells to mutagenizing conditions to produce mutagenized cells;

(d) culturing said mutagenized cells under conditions in which cell survival is dependent upon autocrine growth factor production;

(e) recovering progeny cells that survive said culturing step;

(f) screening said progeny cells to identify cells that produce a ligand for said orphan receptor; and

(g) preparing polynucleotide molecules encoding said ligand from said identified cells.

13. A method according to claim 12 wherein said polynucleotide is cDNA.

14. A method according to claim 12 wherein said polynucleotide is mRNA.

15. A method according to claim 12 wherein said polynucleotide is genomic DNA.

16. A method according to claim 12 wherein said parent cells do not express detectable levels of said orphan receptor.

17. A method according to claim 12 wherein, prior to said culturing step, said mutagenized cells are cultured in the presence of said exogenous growth factor.

18. A method according to claim 12 wherein said screening step comprises assaying media conditioned by said

progeny cells for growth-promoting activity on said transfected cells.

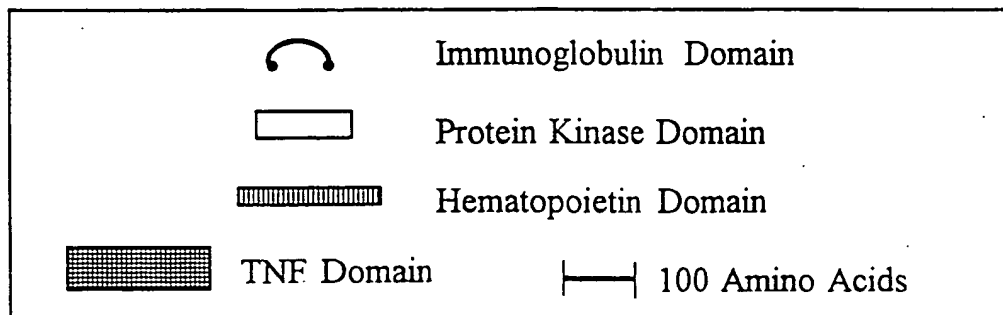
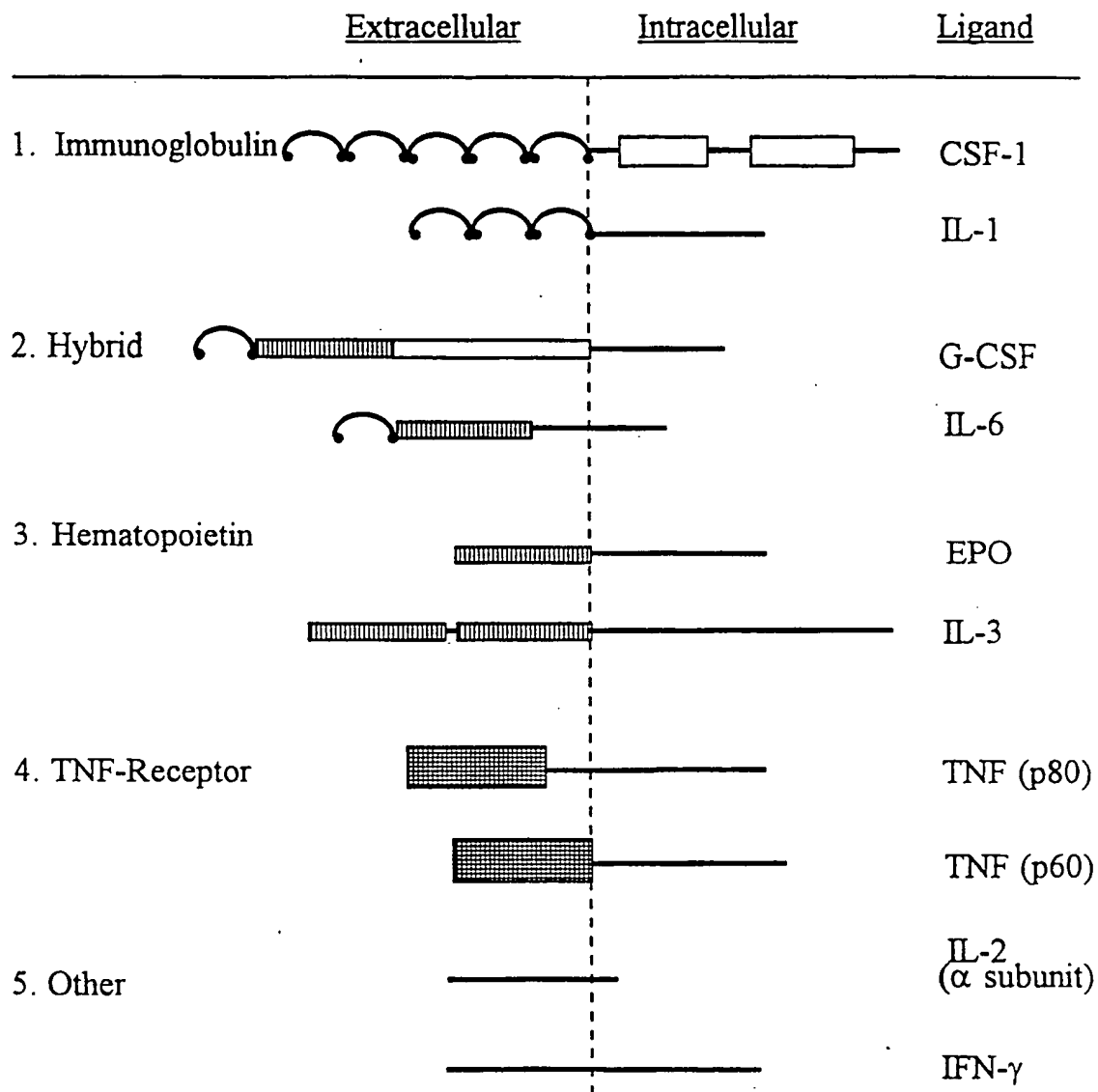
19. A method according to claim 18 wherein said assay is carried out in the presence of an antibody to a known growth factor.

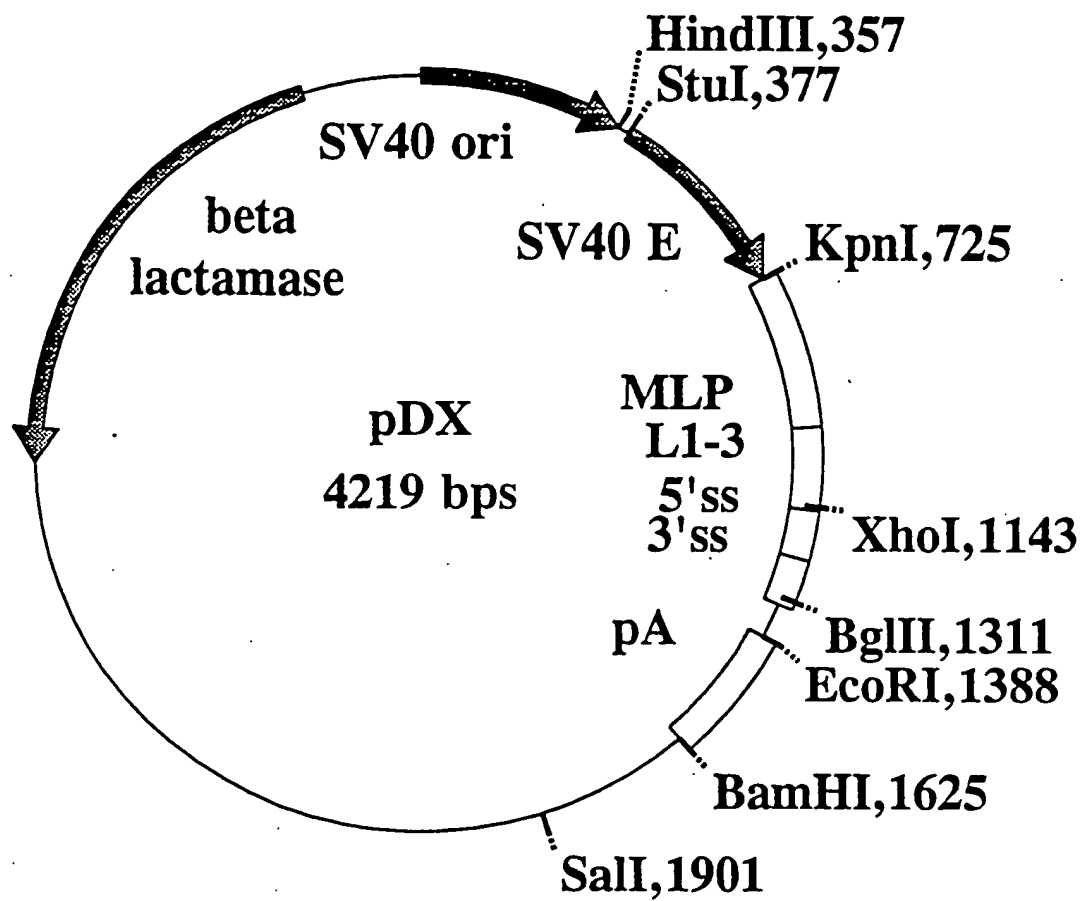
20. A method according to claim 18 wherein said assay is carried out in the presence of a soluble form of said orphan receptor.

# Figure 1

## Receptor Family

## Receptor Structure



**FIGURE 2**

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